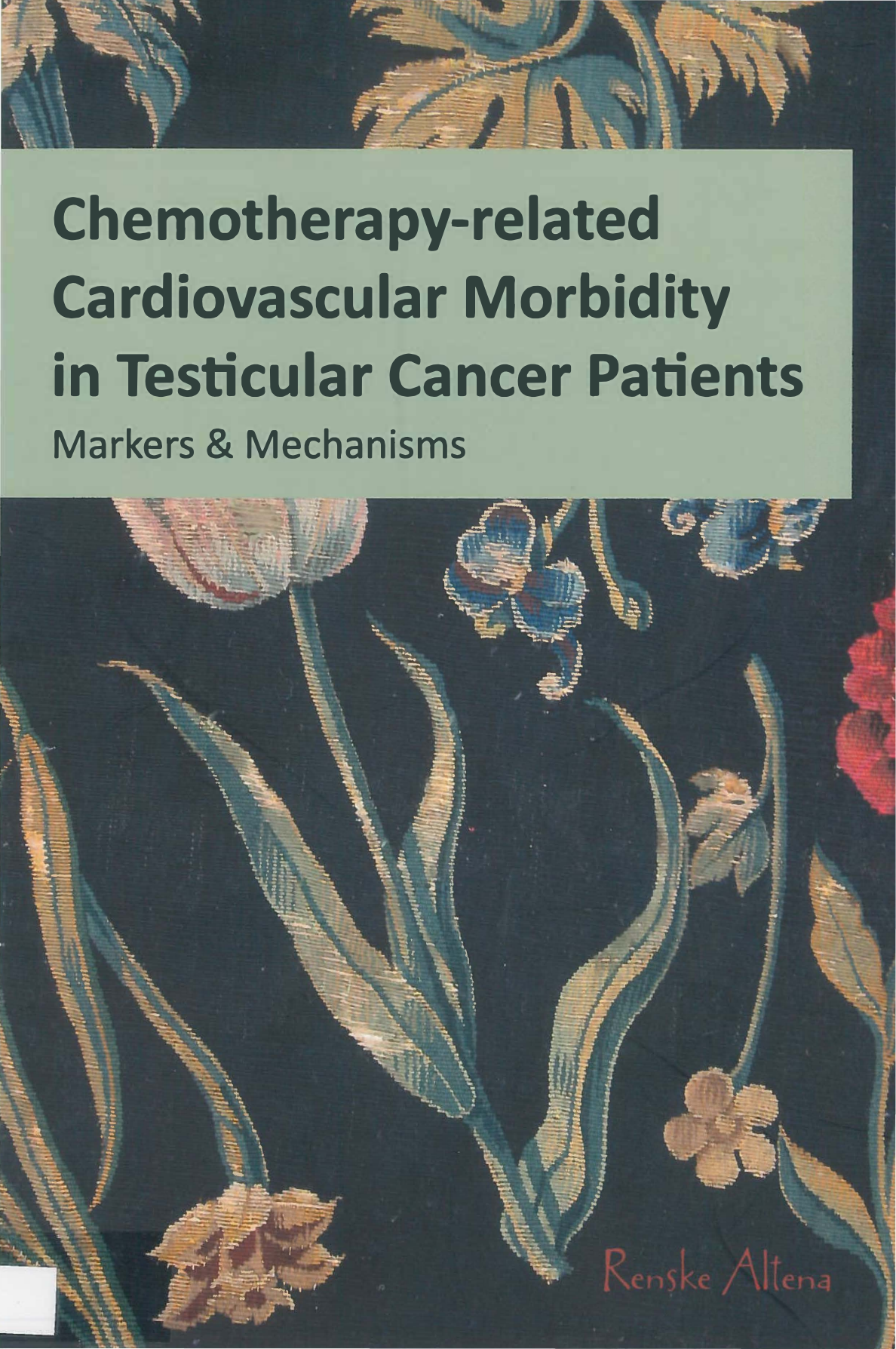
Chemotherapy-related Cardiovascular Morbidity in Testicular Cancer Patients

Markers & Mechanisms



Renske Altena

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De weg bestaat in het begaan ervan

Zhuang Zi

CONTENTS

Introduction and outline of the thesis

Chapter 1

Testicular germ cell tumours, the most common solid malignancy in men in their 2nd to 4th decades, are diagnosed in around 700 patients each year in the Netherlands.[1] Initial management consists of orchidectomy and evaluation for metastases by measurement of serum tumour markers and imaging of the abdomen and thorax. Without signs of metastases at the moment of orchidectomy, a watchful wait-and-see policy is initiated. If metastases are present, or when they arise during follow-up, systemic treatment with platinum-based chemotherapy is indicated.[2] Currently, the standard regimen consists of three or four 3-weekly cycles of Bleomcyin, Etoposide and Cisplatin (BEP) combination chemotherapy. Classification into a prognosis group [good, intermediate or poor International Germ Cell Cancer Collaboration Group (IGCCCG)] is based on levels of the serum tumour markers aFP, bHCG and LDH,[3] and dictates the number of chemotherapy courses. Stage of disease is based on size and sites of distant metastases.

Since the introduction of platinum-based chemotherapy regimens in the late 1970's,[4] metastatic testicular cancer has become the paradigm of a highly curable malignancy with a 10-year survival rate of around 80%.[2,5] As a result of this favourable prognosis, together with the increasing incidence of testicular cancer in the Western world,[1,5] the population of successfully treated testicular cancer survivors is steadily growing (Figure 1).

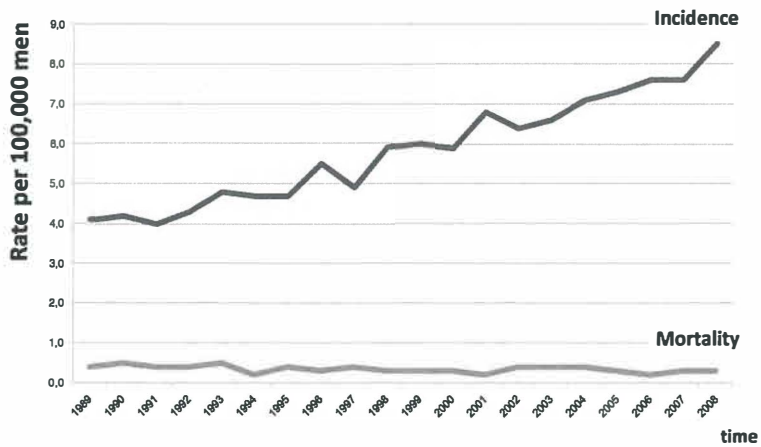

Figure 1. Incidence and mortality of testicular cancer in the Netherlands over the past three decades (data from the Netherlands Cancer Registry, www.ikcnet.nl)

Survivorship issues: chemotherapy-related cardiovascular morbidity

As testicular cancer survivors are thought to have a normal life-expectancy once cured from their malignancy, the importance of the risk for treatment-related complications in these patients is now increasingly recognised.[2,6] Important examples of these late effects are infertility, neurotoxicity, second malignancies and cardiovascular disease (CVD).[6,7] Recognition and possibly prevention of these issues is needed to achieve an optimal health status of the cancer survivor population. The development of CVD is a gradual process that progresses from subclinical changes to clinical morbidity, and therefore, early interventions might be effective to slow down or stop development towards overt CVD.

The time of occurrence of vascular damage and complications related to chemotherapeutic treatment in testicular cancer patients has two peaks; acute toxicity can manifest during of shortly after chemotherapy, whereas chronic consequences can arise up to decades after

completion of treatment. During or shortly after completion of treatment, arterial and venous thrombo-embolic events occur in around 10% of patients treated with BEP-chemotherapy.[8,9] As a more subtle sign of vascular toxicity, over 30% of testicular patients report Raynaud's phenomenon after treatment with bleomycin- and platinum-containing chemotherapy.[10] Bleomycin-induced pneumonitis occurs in 0-10% of patients treated with bleomycin-containing chemotherapy, depending on the criteria used for the diagnosis, and is fatal in approximately 1-3% of all patients treated with bleomycin.[11]

Long-term testicular cancer survivors have a higher incidence of CVD compared to the general population and stage I patients, treated with orchidectomy only.[7,12-14] In a cohort of >2,500 Dutch testicular cancer survivors, the estimated 20-year risk for CVD was 18.1%.[15] In a Norwegian cohort of long-term testicular cancer survivors, treatment with BEP-chemotherapy resulted in a 5.7-fold higher risk (95% CI 1.9-17.1) for coronary artery disease, compared to stage I patients.[16] In this cohort, 8.0% of patients had developed one or more atherosclerotic events at median 21 years of follow-up. In 10-year testicular cancer survivors in the United Kingdom, treatment with chemotherapy was associated with a relative risk of 2.59 for cardiac events compared to stage I testicular cancer patients and healthy subjects.[12] From a previous study in 14-year chemotherapy-treated testicular cancer survivors from our own institution, 6% had developed an ischemic cardiac event, with an observed-to-expected ratio of 7.1 (95% CI 1.9-18.3) compared to the general population.[13] Moreover, cisplatin-based chemotherapy is associated with an elevated risk for cardiovascular mortality, with an standardized mortality rate of 1.20 (95% CI 1.00-1.50) compared to the general population.[17]

Next to overt CVD, testicular cancer survivors have a higher rate of cardiovascular risk factors like dyslipidemia, obesity, hypertension and insulin resistance, compared to healthy controls and stage I testicular cancer patients.[18,19] Moreover, they have an enhanced rate of early signs of atherosclerosis, such as albuminuria and elevated inflammatory markers when compared to controls.[19-21] These subclinical signs of vascular dysfunction may progress to overt CVD over the course of years. This hypothesis was supported by a recent study in a Norwegian cohort of long-term chemotherapy-treated testicular cancer survivors, where higher levels of systemic inflammation (i.e. plasma levels of high-sensitivity C-reactive protein) were associated with a 2.79 fold (95% CI 1.22-6.34) higher risk for cardiovascular events during follow-up.

Mechanisms contributing to chemotherapy-related cardiovascular damage

Different mechanisms have been associated with the development of treatment-related cardiovascular complications in testicular cancer patients. In vitro studies show that both cisplatin and bleomycin can induce endothelial cell death.[22-24] In addition, these drugs change endothelial cell function, including the increased expression of adhesion molecules,[24-26] production of pro-inflammatory cytokines[27] and induction of pro-coagulant activitity.[22,24] Early signs for vascular damage, e.g. rises in endothelial activation markers and an increase in Intima Media Thickness (IMT) of the carotid artery, were previously reported in testicular cancer patients treated with cisplatin-based chemotherapy.[8] Chronic low-grade vascular damage may persist on the long term, as testicular cancer survivors were found to have levels of circulating platinum from years to decades after chemotherapy.[28] Over the course of years, this endothelial damage may progress to accelerated atherosclerosis and overt CVD.[29,30]

In addition to direct chemotherapy-induced endothelial damage, other factors can contribute to the development of vascular complications after testicular cancer treatment. Firstly, the development of cardiovascular risk factors, which have a higher prevalence in chemotherapy-treated testicular cancer patients than in controls,[18,19] can lead to accelerated atherosclerosis and secondary vascular complications. Furthermore, several metabolic changes are associated with the development of an unfavourable risk profile for CVD.[31] In testicular cancer patients, (subclinical) hypogonadism related to unilateral orchidectomy, might further contribute to the development of cardiovascular risk factors, as low testosterone levels are associated with metabolic disturbances and thereby an increased risk for CVD in the general male population.[32] Additional metabolic changes related to cancer treatment, e.g. obesity, dyslipidemia and hypothyroidism, may contribute to the development of CVD in successfully treated testicular cancer survivors.[18,19,31]

Early biomarkers for vascular damage in testicular cancer patients: a potential strategy to reduce the development of cardiovascular complications

When testicular cancer survivors develop cardiovascular risk factors or overt CVD, treatment recommendations based on guidelines from general cardiovascular medicine for initiation of primary or secondary prevention apply. However, patients at risk for these complications should ideally be identified prior to the development of overt morbidity, in order to avoid the development of irreversible complications. The occurrence of CVD is a gradual process that progresses from subclinical damage to clinical morbidity. Therefore, early biomarkers may aid to identify patients at increased risk for vascular complications after chemotherapy. Different types of cardiovascular biomarkers can be discerned for this purpose, as summarised in Table 1.[33]

In cardiovascular medicine, the use of biomarkers for diagnostic, interventional and prognostic purposes is widely studied and increasingly used in clinical practice. A recent definition from the National Institutes of Health described a biomarker as 'a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention'.[34] By the identification of biomarkers for chemotherapy-related cardiovascular damage, treatment and follow-up schedules for patients can be optimised and individualised. In addition, specific biomarkers may increase understanding in the pathogenesis of vascular complications, and thereby provide possible targets for intervention. Furthermore, biomarkers can serve as surrogate markers for disease pro- or regression, and thereby facilitate studies that are aimed to decrease the incidence of chemotherapy-related CVD, e.g. intervention studies.

VASCULAR BIOMARKER	REPRESENTS	EXAMPLE
Circulating markers	*Vascular vulnerability*	- Lipid profile (cholesterol, high-densitiy/low-density lipoproteins, triglycerides) - Systemic inflammation (high-sensitivity C-Reactive Protein, fibrinogen) - Endothelial activation (von Willebrand Factor, soluble adhesion molecules) - Direct endothelial damage (Circulating Endothelial Cells)
	Blood vulnerability	- Pro-thrombotic state (tissue Plasminogen Activator, Plasminogen Activator Inhibitor type 1, von Willebrand Factor, fibrinogen) - Repair capacity (Endothelial progenitor cells)
	Cardiac biomarkers	- Elevated filling pressures (atrial and brain natriuretic peptides) - Direct cardiomyocyte damage (troponin I and T) - Cardiac fibrosis (galectin-3)
Structural marker	*Subclinical atherosclerosis*	- Carotid artery Intima Media Thickness - Coronary calcification score
Functional marker	*Endothelial function*	- Blood pressure - Endothelial dysfunction (Flow-mediated vasodilatation, brachial artery reactivity, carotid artery compliance and distensibility) - Microalbuminuria

Table 1. Cardiovascular biomarkers represent (combinations of) different aspects of cardiovascular pathophysiology, and may aid in early detection of cardiovascular damage related to cancer treatments. Adapted from Vasan, Circulation 2006.[33]

AIM OF THE THESIS

The studies described in this thesis aim to search for early biomarkers of subclinical treatment-related cardiovascular damage in testicular cancer patients treated with cisplatin-based chemotherapy.

OUTLINE OF THE THESIS

Different strategies can be used to evaluate treatment-related cardiovascular damage during or after cancer treatments. Early changes may point to subclinical cardiovascular damage prior to the development of clinical morbidity. In **Chapter 2** conventional and contemporary methods for early detection of cardiovascular damage related to cancer treatments are summarised and graded based on their Level of Evidence.[35] The emphasis is on detection of treatment-related cardiotoxicity, as most studies so far have focussed on this type of damage.

Previously, early changes in endothelial activation and vascular structure in testicular cancer patients shortly after cisplatin-based chemotherapy were described.[8] In **Chapter 3** changes in a comprehensive panel of vascular biomarkers are measured, including Circulating Endothelial

Cells in blood, and plasma markers for endothelial activation, inflammation and pro-thrombotic state. Levels of these markers are prospectively assessed in a cohort of testicular cancer patients before, during and up to one year after completion of cisplatin-based combination chemotherapy. Changes in biomarkers are related to early clinical cardiovascular events and changes in vascular structure.

Cardiac abnormalities, particularly diastolic dysfunction, are found more frequently in chemotherapy-treated long-term testicular cancer survivors, when compared to healthy controls and stage I testicular cancer patients.[13,36] In **Chapter 4** early changes in systolic and diastolic echocardiography parameters and serum levels of the cardiac biomarker N-terminal Pro-Brain Natriuretic Peptide are assessed in a cohort of testicular cancer patients, before start of chemotherapy and within median 10 months after completion of treatment. Baseline measurements are compared to age-matched healthy males. In **Chapter 5** longitudinal follow-up of cardiac status in this cohort of testicular cancer survivors is described. Changes in echocardiography parameters and cardiac biomarkers at median 7 years post-treatment are related to earlier cardiac assessments and patient characteristics.

Long-term testicular cancer survivors treated with chemotherapy have an higher rate of endothelial dysfunction[21] and overt CVD,[13] compared to healthy controls and stage I testicular cancer patients. In **Chapter 6** longitudinal follow-up of vascular status is presented in a cohort of testicular cancer patients median 7 years after treatment with cisplatin-based chemotherapy. The progression of subclinical signs for atherosclerosis over time is estimated, and factors that contribute to the progression of vascular dysfunction are explored.

Chemotherapy-treated testicular cancer survivors have a higher incidence of the metabolic syndrome, a cluster of unfavourable cardiovascular risk factors.[19,21] In **Chapter 7** the prevalence and timing of the metabolic syndrome is investigated, and associated features of vascular status, as well as of circulating vascular biomarkers in testicular cancer survivors with the metabolic syndrome are explored.

Both cisplatin and bleomycin can induce endothelial cell activation and apoptosis,[24-27] which contributes to the development of CVD in testicular cancer patients. Insight in molecular events may provide enhanced understanding and identification of biomarkers for chemotherapy-related endothelial damage. In **Chapter 8** preliminary results from cDNA microarray experiments performed in an endothelial cell line model exposed to cisplatin or bleomycin are described. Findings from this pre-clinical study are translated to a clinical setting by measurement of candidate biomarker levels in testicular cancer patient plasma samples.

Data from this thesis are summarised in **Chapter 9**, and potential strategies that can be pursued to reduce chemotherapy-related endothelial damage are discussed. Findings from the current work are put into perspective according to these potential strategies, and recommendations for future research are presented.

Cardiovascular toxicity caused by cancer treatment: strategies for early detection

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Chapter 2

SUMMARY

Cardiovascular toxicity is one of the most devastating complications of cancer treatment and can arise during or shortly after treatment, or even several years later. Identification of the left ventricular ejection fraction (LVEF) is the most common method to screen for toxic effects on the heart; however, this approach underestimates cardiac damage and additional strategies for the monitoring of treatment-induced cardiotoxicity are being explored. Guidelines for monitoring have been formulated for several cancer treatments; however, appropriate underlying evidence is still largely absent. In this Review, we summarise conventional and contemporary methods for early detection of cardiotoxicity and designate a level of evidence for the basis of each method.

INTRODUCTION

Methods for detection and treatment of different types of cancer have developed impressively in the past 20 years. Life-expectancy for patients with cancer is steadily improving, with an age-adjusted 10-year survival of 70% for breast cancer, 80% for Hodgkin's disease, and 90% for testicular cancer.[5] Around 3 million patients are diagnosed with cancer in Europe each year, which means there is a large group at risk of treatment-related complications.[37] Long-term complications include development of second malignancies and cardiovascular disease, but up to now, the incidence and extent of these complications in adults are largely unknown. By contrast, studies of childhood cancer survivors have produced many more data on long-term complications of treatment.[38]

In trials of adjuvant anthracycline-based treatment for breast cancer, 5-year incidence of chronic heart failure is between 0% and 3.2%, depending on the combination regimen and cumulative dose of anthracycline.[39] However, long-term follow up of cardiovascular morbidity and mortality is not available. A large meta-analysis of the Early Breast Cancer Trialists' Collaborative Group, in which 42,000 patients with breast cancer participated in 78 randomised trials, found that patients treated with radiotherapy had a high rate of non-breast cancer mortality, mainly from heart-disease (rate ratio 1.12). This high rate of heart disease was observed during the first 5 years after treatment and continued for up to 15 years.[40] However, data are partly based on outdated radiation-techniques in use from 1976 until 1995.

10 years ago, we reviewed methods for detection of anthracycline-induced cardiotoxicity.[41] Improvements have since resulted in more sensitive monitoring strategies (Figure 1). Changes in cancer treatment have introduced both, more as well as less, potentially cardiotoxic regimens, through the increased use of anthracyclines, platinum compounds, radiation therapy, and introduction of monoclonal antibodies such as trastuzumab and bevacizumab.

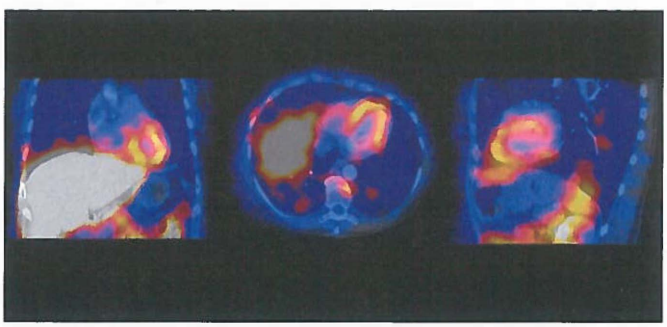

Figure 1. Fused computed tomography with 111Indium-DTPA-trastuzumab single photon emission tomography (SPECT) image performed in a patient with metastatic osteosarcoma three weeks after 6 cycles after completion of doxorubicin (96 hours post tracer injection). Fusion of the images shows increased uptake of the tracer in the characteristic horseshoe shape of the myocardium of the left ventricle.

Although monitoring guidelines exist for several potentially cardiotoxic treatments, little data are available to formulate evidence-based screening and follow-up recommendations for treatment-induced cardiotoxicity. In 2007, the American Society of Clinical Oncology (ASCO) Survivorship Expert Panel clearly stated the need for such recommendations.[42]

In this Review, we give an update of the methods available for early detection of cardiac damage caused by cancer treatment. Each method is assigned a level of evidence backing up its applicability, according to the system used by the Oxford Centre of Evidence Based

Medicine (Table 1).[35] Level 1a evidence is viewed as definite and level 5 as weak. Most studies reviewed in this paper were not designed to assess the sensitivity of monitoring strategies as a primary goal, which implies that level of evidence is based on indirect evidence or retrospective data.

Level	1	*a*	Systematic review of randomised controlled trials
		b	Single randomised controlled trial
	2	*a*	Systematic review of cohort studies
		b	Individual cohort study
		c	"Outcomes" research
	3	*a*	Systematic review of Case-Control studies
		b	Individual Case-Control study
	4		Case-series
	5		Expert opinion

Table 1. Levels of Evidence according to the Oxford Center of Evidence Based medicine. [35]

Detection of treatment-induced cardiotoxicity

Cardiac function is monitored during a potentially cardiotoxic cancer treatment to identify patients who are susceptible to this toxicity as early as possible and prevent morbidity and mortality. Efforts to prevent further complications include intensified monitoring, initiation of preventive measures, or changes to cancer treatments. Detection of possible toxicity should not result in holding back an essential treatment, which would compromise effectiveness.

Mitani and colleagues[43] did a retrospective analysis of 265 patients treated with doxorubicin who had serial assessments of left ventricular ejection fraction (LVEF) during treatment. Changes in LVEF were associated with chronic heart failure up to 3 years after treatment. Patients were identified as being at risk (decrease in LVEF of >10% to <50%) or at low risk of cardiotoxicity. 15 cases of chronic heart failure were prevented by stopping cancer treatment when patients met the criteria for being at risk. Mitani and colleagues[43] concluded that monitoring of cardiac function to identify at-risk patients was effective in preventing progression to chronic heart failure.

The possibility of preventing cardiotoxicity should be viewed in light of possible loss of benefit to cancer outcome, although it is unclear how this issue should be interpreted. This is especially difficult for trastuzumab-induced cardiotoxicity because data on long-term outcome are unavailable.

Several guidelines for monitoring of cardiovascular effects during and after cancer treatment are available. The American Heart Association (AHA) recommends close monitoring of cardiac function during anthracycline treatment but does not specify how often or by which means.[44] The absence of data on the role of routine monitoring to prevent cardiac disease in asymptomatic adult survivors of cancer was emphasised in a recent ASCO clinical evidence review.[42] For survivors of childhood cancer, the Children's Oncology Group has devised risk-based guidelines for follow-up of late effects, such as cardiac morbidity.[45] Available guidelines are not consistent in their recommendations and seem to be based on modest evidence.

Moreover, none of the guidelines define an approach to long-term follow-up of cardiac function in adult survivors of cancer, although cardiac morbidity can become apparent up to several years after treatment.

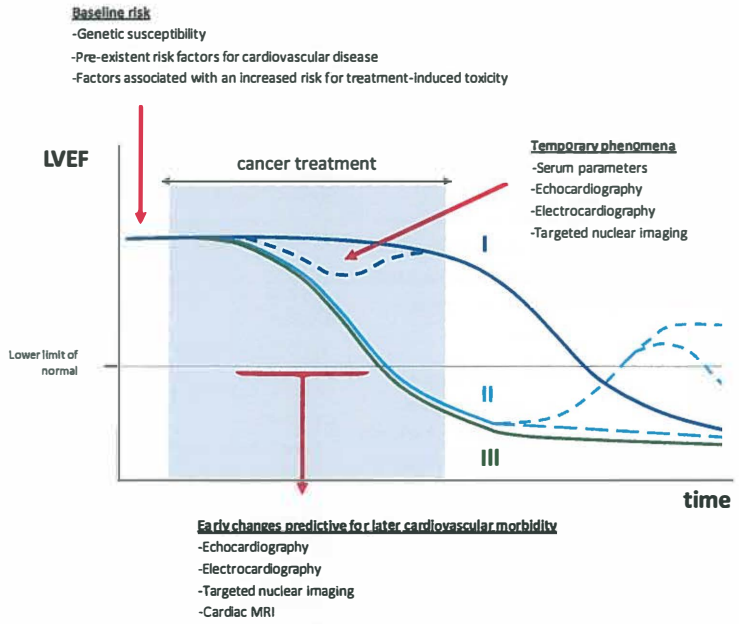

Figure 2. Schematic, hypothetical representation of changes in left ventricular ejection fraction (LVEF) and timing of detection with several screening strategies during and after cancer treatment. (I) late onset cardiotoxicity (e.g. radiotherapy, anthracyclines);[46,47] (II) reversible cardiotoxicity (e.g. trastuzumab: dotted line as long-term consequences are still unknown);[48] (III) irreversible cardiotoxicity during treatment (e.g. anthracyclines).[46,49]

LVEF

Measurement of LVEF with multiple gated acquisition (MUGA) scintigraphy and echocardiography,[50] gives an assessment of systolic cardiac function and is the most common method of monitoring cardiac function during cancer treatment. However, LVEF can underestimate actual cardiac damage[51] because of the compensatory reserve of the myocardium that enables adequate ventricular output even in the presence of dysfunctional myocytes. With left ventricular dysfunction, deterioration of diastolic cardiac function can be present in the absence of systolic impairment, and subclinical diastolic dysfunction precedes a drop in systolic function in many patients.[52] Thus, adequate LVEF can mask actual cardiac damage and therefore other methods to assess cardiac function during cancer treatment are being investigated.[53] Several approaches towards early detection of cardiovascular toxicity might be useful for this purpose, including estimation of the baseline risk of cardiovascular complications, detection of temporary events (eg, release of troponins or natriuretic peptides), or identification of subclinical changes, such as changes in diastolic function (Figure 2).[54-57] Decreases in LVEF resulting from treatment might vary with different types of treatment, hindering the interpretation of cardiac function assessments.

Although guidelines are available for the monitoring of cardiovascular effects during and after cancer treatment, the underlying evidence is of medium quality. Furthermore, the time after completion of treatment for which cardiovascular follow-up is needed is unknown.

Methods for assessment of cardiovascular function

Strategies for early detection of potential cardiovascular damage from cancer treatment will be addressed. Advantages, disadvantages, and level of evidence behind each strategy are summarised in Table 2.

	Advantages	Disadvantages	Level of evidence
Multiple Gated Acquisition Scintigraphy	- Reliable calculation of Left Ventricular Ejection Fraction (LVEF) - Low intra-individual and inter-observer variation	- LVEF insensitive for early, subclinical cardiotoxicity - Limited information on diastolic function - Radiation exposure	*2b*
Echocardiography	- No side effects, non-invasive - Systolic and diastolic function - Tissue Velocity Imaging, Strain and Strain Rate promising for detecting early (subclinical) changes	- Preload-dependence of several parameters - Expertise and interpretation of echocardiographist	*2b*
Electrocardiography	- Availability - No side effects, non-invasive - Computerized analysis enables standardized serial assessment - Prolonging QTc-time may be an early marker	- No information on left ventricular function - QTc only investigated parameter - Variation in inter-observer interpretation - Timing of ECG changes in the course of cardiotoxicity unknown - Interference with other medication	*2b*
Biochemical markers	- Easy analysis, possible quantification - Minimal invasive - Availability - Low inter-observer variability - Rises in levels probably relate to early toxicity	- Exact predictive value not certain - Normal / abnormal values not definite	*2b*
Endothelial damage	- Pathogenic factor in toxicity - Assessment of structural and functional parameters - Rises in serum/plasma levels possibly relate to early toxicity - Identification of cardiac and vascular toxicity	- Exact predictive value not certain - Unknown which marker reliably represents cardiovascular toxicity - Intra-individual variation of functional parameters	*2b*
Genetic variations	- Pre-treatment identification of susceptibility to toxicity - Minimal invasive	- Numerous SNPs, difficulty of identifying most relevant	*4*
Targeted cardiac imaging	- Information on structural features - Information on functional and/or metabolic processes - Early changes in metabolic processes can be visualized	- Availability - Costs - Radiation exposure - Exact predictive value not certain - Mainly older data	*2b*

Table 2 - continued

	Advantages	Disadvantages	Level of evidence
Cardiac Magnetic Resonance Imaging	- Detailed information of anatomy with high resolution and contrast - No radiation exposure - Reliable calculation of LVEF - Combination with late gadolinium enhancement for detailed information on myocardial function	- Availability - Costs - Unknown whether early cardiac changes can be visualized - Contra-indicated with metal implants	*4*

Table 2. Strategies for early detection of cancer treatment-induced cardiovascular toxicity; overview of (dis-) advantages and levels of evidence.

MUGA scintigraphy

With high reproducibility and low interobserver and intraindividual variability, MUGA scintigraphy is the gold standard for assessing LVEF. Together with echocardiography MUGA scintigraphy is the most widely accepted method to assess patients' LVEF during cancer treatment;[50] although guidelines do not specify which is preferred.

In a combined analysis of three phase III trials, with a total of 630 patients treated with doxorubicin, serial measurements of LVEF were retrospectively analysed and compared with clinical symptoms of cardiotoxicity. During treatment, 149 patients were judged to have a decrease in LVEF (>30% from baseline), whereas 32 patients developed chronic heart failure during follow-up. Only 11 of the 32 patients with chronic heart failure had a preceding decrease in LVEF. The authors concluded that early changes in LVEF are inadequate to predict anthracycline-induced heart failure.[54]

A prospective study of 120 patients with breast cancer recorded LVEF up to 3 years after treatment with anthracycline. 11 of the 12 most severe reductions in LVEF (decrease of >35% from baseline) happened more than 3 months after treatment stopped, and cardiac systolic function showed a slowly progressive deterioration in the 3 years of follow up.[55] This study indicates that prolonged monitoring of cardiac function after stopping of treatment is necessary to detect treatment-related cardiotoxicity, and monitoring of LVEF is insensitive to early changes in cardiac function during a potentially cardiotoxic treatment.

Nousiainen and co-workers[56] studied 28 patients treated with doxorubicin for non-Hodgkin lymphoma who had successive LVEF assessments at increasing cumulative doses. A MUGA scan showing a decrease in LVEF of at least 4% after a cumulative doxorubicin dose of 200 mg/m^2 had 90% sensitivity and 72% specificity for predicting development of chronic heart failure.[56]

MUGA scintigraphy is a reliable and widely used method for assessment of LVEF, but it is somewhat insensitive for detecting subtle changes in cardiac function. Therefore, MUGA scintigraphy has limited value for early detection of cardiotoxicity. Nousiainen and co-workers[56] small prospective study found early changes on MUGA scintigraphy predictive for later development of chronic heart failure, but a large retrospective analysis identified no association between clinical findings and LVEF.[54] Changes in LVEF indicative of cardiac damage can only be identified when serial measurements are compared and related to baseline ejection fraction. This should be taken into account because the LVEF has a broad range of values in healthy individuals, which can lead to masking or overestimation of possible cardiotoxicity.

Echocardiography

Echocardiography allows assessment of systolic and diastolic cardiac function. Diastolic measurements are probably the most sensitive to early changes in cardiac function. Commonly used diastolic measurements include the E/A ratio (peak early atrial divided by peak late atrial velocities) and recent techniques such as tissue velocity imaging of the early diastole, strain, and strain rate. These contemporary techniques are reliable measures of diastolic function because they are less subject to interobserver variation than the E/A-ratio.[57,58]

Several studies of early changes in cardiac function during and after anthracycline treatment found primarily diastolic function impairment, rather than systolic dysfunction.[59-61] Diastolic impairment was also observed in 16 patients with breast cancer within 24 h of first high dose of cyclophosphamide.[62] In a prospective study of 16 patients treated with anthracycline, the mean strain and strain rate decreased after low cumulative doses of epirubicin (<200 mg/m^2), without changes in other systolic or diastolic echocardiographic variables.[63] Other small prospective studies compared conventional echocardiography with doppler echocardiography, which assesses blood-flow velocities and myocardial structures, during and shortly after anthracycline-treatment. In these studies, doppler imaging enabled enhanced detection of subclinical cardiac changes.[59,64,65]

Lotrionte and colleagues[66] are investigating doppler echocardiography for detecting changes in systolic and diastolic measures as primary endpoints for cardiotoxicity during treatment with anthracycline. Data from this randomised clinical trial, in which patients are given doxorubicin or liposomal doxorubicin, will hopefully help identify measures that are sensitive to early changes in cardiac function during cancer treatment.

Echocardiography is mainly used to assess changes in LVEF during cancer treatment. Several reports indicate that newer diastolic measures such as tissue velocity imaging of the early diastole, strain, and strain rate enable early detection of subclinical changes in cardiac function during cancer treatment,[59,63-65] although their predictive value is unclear.

Electrocardiography

Electrocardiography (ECG) is a convenient and inexpensive method to screen for conduction disturbances and signs of cardiomyopathy. Several chemotherapeutics can induce cardiac problems that might lead to changes in an ECG, such as repolarisation abnormalities and prolongation of the corrected QT interval. Cardiomyopathy is indicated on an ECG by low QRS voltages.[50]

Most prospective studies on the role of ECG in screening for cardiotoxicity measured changes in the corrected QT interval, possibly because this variable is easily assessed and compared. Corrected QT interval was prolonged with each successive course of anthracycline-based treatment in 21 adults, but no information was given on long-term cardiac function in this group.[67] Nakamae and co-workers[68] showed a long corrected QT interval to be predictive of subacute (potentially fatal) cardiac failure in 19 patients who received high-dose cyclophosphamide. The same authors found an association between prolonged corrected QT interval and decline in systolic function in 72 adults after treatment with anthracycline,[69] suggesting that a prolonging corrected QT interval might indicate cardiotoxicity. Large studies of the predictive value of changes in corrected QT interval have not been done. Other drugs used in supportive care can also affect the corrected QT interval and hinder its interpretation.

Few data are available on changes in other ECG variables. Decreases in QRS voltage within 24h were observed in six of 16 patients with breast cancer given high-dose cyclophosphamide, but follow-up longer than 24 h was not provided.[62] Little evidence is available to define a role for ECG in the assessment of potential cardiotoxicity. Several individual cohort studies suggest that a prolonging corrected QT interval is an early marker,[68,69] but the accuracy for prediction of late cardiac disease is not established.

Biochemical cardiac markers

Measurement of serum biomarkers is an attractive strategy for the monitoring of cardiotoxicity because samples can be taken in a minimally invasive way and there is low interobserver variability in their interpretation. Natriuretic peptides (n-terminal pro-brain natriuretic peptide, brain natriuretic peptide, and atrial natriuretic peptide) are used as predictive markers for severity of heart failure,[70] and they are released from cardiomyocytes in response to increased ventricular wall stretch. Concentrations of cardiac troponins T and I relate to the extent of acute myocardial damage.[71] Concentrations of plasma atrial natriuretic and brain natriuretic peptide were prospectively assessed in 13 adults with leukaemia during doxorubicin treatment and related to LVEF. Three patients developed chronic heart failure and five developed an asymptomatic LVEF decrease after chemotherapy. In all patients with cardiac dysfunction, the concentrations of plasma brain natriuretic peptide increased above the normal limit before the LVEF deteriorated, whereas this did not change in patients without cardiotoxicity. Concentrations of plasma atrial natriuretic peptide were unrelated to changes in LVEF.[72]

In 27 adults treated with anthracycline for different types of malignancy, N-terminal pro B-type natriuretic peptide was increased without clinical signs of cardiotoxicity.[73] Increased concentrations of natriuretic peptides were not associated with changes in LVEF in a prospective study of 40 patients with breast cancer treated with anthracycline.[74] Mean N-terminal pro B-type natriuretic peptide concentrations were higher after 24 h in 21 adults given anthracycline than in those who were not, without changes in serum concentrations of cardiac troponin T.[67] Sandri and colleagues[75] assessed concentrations of N-terminal pro B-type natriuretic peptide in 52 adults undergoing high-dose chemotherapy for various malignant diseases. Patients with persistently high concentrations of N-terminal pro B-type natriuretic peptide had more cardiac (systolic and diastolic) dysfunction 1 year later than did patients with transient increases or stable concentrations.[75]

Serum concentrations of cardiac troponin T and N-terminal pro B-type natriuretic peptide were prospectively assessed by Dodos and co-workers[76] and compared with changes in LVEF in 100 adults treated with anthracycline. Only minor increases in cardiac troponin T concentrations were found and transient increases in N-terminal pro B-type natriuretic peptide were seen in 15 patients after the first cycle of anthracycline, which was not associated with cardiac dysfunction.[76]

In a prospective study of 703 adults, cardiac troponin I concentrations were assessed immediately after and 1 month after completion of high-dose chemotherapy regimens for different tumour types. Patients with high cardiac troponin I concentrations at either assessment had a high incidence of cardiac events (37% at one assessment time, 84% at both times) during 3 years follow-up compared with those without raised troponin I.[77]

A predictive role for biomarkers of cardiotoxicity caused by cancer treatment is not defined well enough to include them as routine screening measurements, although persistent increases in cardiac troponin I[77] or N-terminal pro B-type natriuretic peptide[75] concentrations seem to identify patients at risk for cardiotoxicity. Therefore, these biomarkers might aid in early detection of subclinical damage. A useful approach is to assess baseline biomarker concentrations in every patient and measure periodically during and after a potentially cardiotoxic cancer treatment. Increases in concentrations of these markers might signal the need for further cardiac assessment.

Markers of endothelial damage

Structural, functional, or biochemical indicators of endothelial damage might be useful markers for early detection of cardiovascular toxic effects. Endothelial activation leads to disturbance of the physiological balance of the endothelium and might progress to endothelial dysfunction and accelerated atherosclerosis.[29,78] Vasoactive factors involved in these endothelial effects include cytokines[27] and adhesion molecules, which might be used as early markers (Figure 3).[25,26,79] Vascular parameters such as carotid intima–media thickness might also be used to characterise potential endothelial damage.

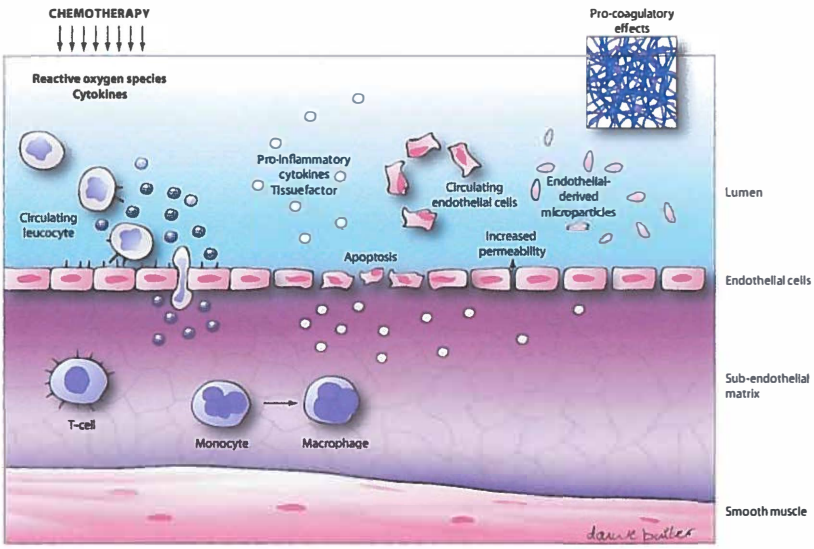

Figure 3. Overview of factors involved in cancer treatment-induced endothelial activation. Several chemotherapeutic agents as well as radiotherapy can initiate a cascade of events, which may ultimately lead to accelerated atherosclerosis.[29] Factors involved in these processes, e.g. adhesion molecules, cytokines, endothelial-derived microparticles or coagulatory and fibrinolytic proteins, may be early markers of endothelial toxicity.

Several clinical studies have analysed endothelial damage during cancer treatment. In 45 patients with testicular cancer who received cisplatin and bleomycin-based regimens, serum concentrations of von Willebrand factor and carotid intima–media thickness were increased 10 weeks after treatment compared with baseline recordings.[8] Circulating endothelial-derived micro particles are thought to have a pro-thrombotic role. A prospective study[80] found high serum concentrations of these microparticles in 18 patients during treatment with cisplatin, suggesting that such increases might contribute to the risk of cerebrovascular events. In

another study,[21] survivors of testicular cancer had microalbuminuria and unbalance in concentrations of the plasma fibrinolytic proteins tissue-type plasminogen activator (tPA) and plasminogen activator inhibitor type 1 (PAI-1) at 7 years after treatment. Additionally, survivors of testicular cancer in another study[20] had high serum concentrations of intercellular adhesion molecule-1 (ICAM-1) 5 years after chemotherapy compared with testicular cancer survivors who had not had chemotherapy. In 40 patients with breast cancer who were treated with anthracycline-based regimens, no association was found between plasma concentrations of apoptosis markers and clinical cardiotoxicity.[81]

Individual cohort studies have indicated variables that suggest subacute treatment-induced endothelial activation (von Willebrand factor and intima–media thickness),[8,82] but the predictive role for these factors is unknown. If proven predictive, such markers of early detection might be used as intermediate endpoints for cardiovascular toxicity in intervention trials.

Genetic variation

Genetic variation might modulate the risk of cardiovascular toxicity from cancer treatment. Pharmacogenomics are increasingly investigated in general cardiology in the search for genetic variations that contribute to the development of cardiovascular disease.

Several investigators aimed to identify single nucleotide polymorphisms as an explanation for differences in sensitivity to cytotoxicity caused by cancer treatment.[83-85] Genome-wide screening and candidate gene methods identified several single nucleotide polymorphisms associated with genetic predisposition for increased sensitivity to chemotherapy. Five single nucleotide polymorphisms were linked with early or late doxorubicin-induced cardiotoxicity in 87 patients and were absent in 363 asymptomatic patients treated with anthracycline (odds ratio 2·0–3·6).[86] The single nucleotide polymorphisms included a variant of the NADPH oxidase subunit NCF4, the His72Tyr polymorphism in the p22phox subunit, and polymorphism in the RAC2 subunit of the same enzyme. The Gly671Val variant of the doxorubicin efflux transporter multidrug resistance protein 1 (MRP1) and the Val1188Glu-Cys1515Tyr haplotype of the functionally similar MRP2 were also associated with cardiotoxicity. These genes are all involved in doxorubicin metabolism and formation of reactive oxygen species, supporting the relevance of this finding.

The most common ERBB2 polymorphism is codon Ile655Val. In 61 patients with breast cancer who were treated with trastuzumab, all treatment-related cardiotoxicity was in patients heterozygous for Ile655Val, whereas no cardiotoxicity was recorded in wildtype Ile655Ile patients.[87]

Although the sensitivity and predictive value of single nucleotide polymorphisms are uncertain, further investigation might enable pretreatment identification of patients with a genetic susceptibility for cardiovascular toxicity, for whom more intensive screening or even preventive strategies should be initiated.

Targeted cardiac imaging

Nuclear techniques can assess structural and functional processes in the heart and might be a sensitive method to detect cardiotoxicity. Apart from MUGA scintigraphy, discussed earlier, a range of other targeted tracers are used in cardiology practice, including 111-indiumantimyosin[88] and 123-iodine-metaiobenzylguanidine.[89]

111-Indium-antimyosin enables in-vivo visualisation of myocardial necrosis, which has been investigated in the setting of anthracycline-induced cardiotoxicity. 17 of 20 patients with breast cancer[90] and 27 of 30 patients with sarcoma[91] had cardiac uptake of 111-indium-antimyosin. In both studies, tracer uptake was increased in patients with low LVEF. In a prospective study of 24 patients treated with anthracycline for various malignant diseases, Valdes and colleagues[92] found tracer uptake in patients without changes in systolic or diastolic function. Uptake was more intense with increasing cumulative dose of anthracycline. In a prospective study of 36 patients treated with doxorubicin, Carrio and co-workers[93] showed that patients with higher antimyosin uptake at intermediate cumulative doses tended to have more severe cardiac functional impairment at maximum cumulative doses. 123-Iodine-metaiobenzylguanidine is used to investigate the functionality of the myocardial adrenergic neurotransmitter system, which can be affected by anthracycline.[94] In two studies of 57 patients treated with anthracycline, tracer-uptake decreased with increasing cumulative doses of anthracycline and preceded changes in LVEF.[93,95]

Other potentially useful nuclear tracers are available. For example, imaging with radiolabelled fatty acids to show myocardial-cell metabolism has been investigated in other specialties of cardiology.[96] Radiolabelling and imaging of molecular directed anticancer drugs is another possibility; ERBB2 expression can be visualised with 111-indium-trastuzumab and single-photon emission CT (SPECT; Figure 1). De Korte and colleagues[97] reported myocardial uptake of tracer in five of ten patients with different types of malignancy within 4 weeks of anthracycline-based treatment. This finding suggests that the presence or even upregulation of ERBB2 soon after anthracycline-treatment might pose a patient at risk of trastuzumab-induced cardiotoxicity. In an earlier study,[98] myocardial uptake of the same tracer was found in only one of 15 patients with breast cancer at a median of 11 months after anthracycline treatment. However, no association with clinical evidence of cardiotoxicity was found. Radiolabelling of molecular targets to visualise pathways involved in treatment related complications could be used to investigate other drugs that potentially cause cardiotoxicity (eg, imatinib or sunitinib).

It is unknown how targeted cardiac imaging can be used to detect early changes in cardiac function during or shortly after cancer treatment. For both 111-indiumantimyosin and 123-iodine-metaiobenzylguanidine, scintigraphy showed associations between tracer uptake and subsequent decreases in LVEF,[90-93,95] but most techniques were investigated over a decade ago, and despite promising preliminary results, these strategies have not been incorporated in standard clinical practice. Contemporary imaging techniques, as shown with radiolabelled trastuzumab, could be used to detect changes in the myocardium.

MRI

MRI is a reliable and reproducible method to assess cardiac structural features, including the coronary arteries and pericardium, and enables consistent calculation of LVEF. MRI combined with late gadolinium contrast enhancement can detect subtle areas of irreversibly damaged myocardium. Therefore, MRI is increasingly useful in cardiology.[99] In a recent case study of ten patients with breast cancer—who had previously received anthracycline and had low LVEF on MUGA scintigraphy or echocardiography during trastuzumab treatment— structural MRI abnormalities throughout the myocardium were suggestive of myocarditis in all patients.[100] As this result suggests, cardiac MRI might be practical for early detection of treatment-related cardiotoxicity, although evidence is still preliminary.

CONCLUSION

The identification of patients who are at an increased risk of cardiovascular damage caused by cancer treatment can be achieved by detection of early changes in cardiovascular function and estimation of a baseline risk of late morbidity (Figure 2). Current guidelines and monitoring strategies for detection of cardiovascular toxicity caused by cancer treatment are mostly derived from medium-level evidence—true even for MUGA scintigraphy (Table 2).

LVEF is the most widely used measure in the monitoring of cardiac toxicity but might underestimate actual cardiac damage because patients with a healthy LVEF can have subclinical changes in cardiac function. On the basis of insights from different types of cardiomyopathy, it is likely that diastolic dysfunction happens before the deterioration of systolic function. Therefore, echocardiography with newly developed measures of diastolic function such as like tissue velocity imaging of the early diastole, strain, and strain rate, might permit earlier detection of subclinical signs of cardiac dysfunction. Serum cardiac biomarkers, such as troponins and natriuretic peptides, have shown promising results to identify patients at risk for later cardiotoxicity and might be a screening strategy for early cardiovascular damage. These biomarkers should be further investigated prospectively. Moreover, cardiac MRI, combined with gadolinium enhancement, is a promising new strategy to screen for potential cardiotoxicity that warrants further investigation.

Care for long-term survivors of cancer is a new part of practice in oncology, with cancer increasingly regarded as a chronic disease. Studies that assess reliable and sensitive monitoring strategies for early detection of cardiovascular toxic effects should be encouraged, so that timely recognition of cancer treatment-related consequences is possible, without compromised antitumour efficacy.

Search strategy and selection criteria

Articles for this Review were found through searches of PubMed and Embase by use of the terms "cardiovascular toxicity", "cardiotoxicity", "cancer treatment", "chemotherapy", "biochemical", "endothelial damage", "genetic variations", "electrocardiography", "echocardiography", "Multiple Gated Acquisition Scan", "Magnetic Resonance Imaging", "Positron Emission Tomography", and "Single Photon Emission Tomography". Trials with highest quality study design were selected. Only studies of adults, written in English, and published between January, 1985, and June, 2008, were included.

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A comprehensive biomarker panel for chemotherapy-related endothelial damage detection in testicular cancer patients

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Submitted

ABSTRACT

Purpose

Chemotherapy-related endothelial damage causes cardiovascular morbidity in testicular cancer patients. Early identification of patients at risk for these effects is needed to guide preventive strategies. We therefore studied changes in a comprehensive panel of biomarkers involved in vascular pathobiology.

Methods

In metastatic testicular cancer patients before, during, 1 month and 1 year after cisplatin-based chemotherapy, CD146+, CD105+, DAPI+, CD45- circulating endothelial cells (CECs) and plasma markers for endothelial activation [von Willebrand Factor (vWF), soluble intra-cellular and vascular adhesion molecule (sICAM-1, sVCAM-1)], inflammation (fibrinogen, high-sensitivity C-reactive protein) and fibrinolysis [plasminogen activator inhibitor type 1, tissue-type plasminogen activator] were measured. Carotid intima media thickness (IMT) was determined before and at both post-treatment visits.

Results

Fourty-one patients were included. Compared to baseline, CECs and endothelial activation markers increased during treatment, and were persistently higher 1 month post-chemotherapy. One year post-treatment CECs had normalized, while endothelial activation markers remained elevated. Markers for inflammation and fibrinolysis increased during treatment and normalized post-chemotherapy. Median IMT did not change. Changes in CECs correlated with vWF and sICAM-1 (rs for areas under the curve=0.63; P=0.001 and rs=0.40; P=0.03), and were larger in 17 patients with increased IMT and five patients with cardiovascular events.

Conclusions

Within this comprehensive panel of biomarkers, changes in CECs and vWF as well as carotid IMT point to testicular cancer patients with augmented endothelial damage. These biomarkers should be included in studies addressing the late effect phenotype of cisplatin-based chemotherapy with accelerated atherosclerosis and cardiovascular morbidity in testicular cancer patients.

INTRODUCTION

Since the introduction of platinum-based chemotherapy, metastatic testicular cancer has become a highly curable disease. In the growing group of these cancer survivors we now also learn the downside of this success, namely chemotherapy-related complications such as the increased risk for cardiovascular disease (CVD).[2,6,7,12,13,101] CVD can manifest during or shortly after treatment,[9,102] as well as years to decades thereafter.[12-14,16,103]

Chemotherapy-related mechanisms contribute to this vascular damage, as pre-clinical data show that both cisplatin and bleomycin induce endothelial cell death.[22-24] Moreover, these drugs change endothelial function, including increased expression of adhesion molecules,[24-26] production of pro-inflammatory cytokines[27] and pro-thrombotic activitity.[22,24] Ultimately, it will be this endothelial activation that progresses to accelerated atherosclerosis and overt CVD over time.

In order to develop cardiovascular preventive strategies in testicular cancer survivors, a more profound insight in the mechanisms and kinetics of vascular complications is needed. In this respect, availability of biomarkers specific for early chemotherapy-related endothelial damage will be critical. Until now, only few reports have investigated this. A study investigated some candidate vascular biomarkers in patients treated with cisplatin-based regimens.[8] Here, the carotid Intima Media Thickness (IMT) increased within 10 weeks post-chemotherapy. Others measured biomarkers for atherosclerosis in long-term testicular cancer survivors.21 In both studies, cisplatin-based chemotherapy was associated with elevated von Willebrand factor (vWF) levels.[8,21] This is of interest as vWF, released from activated endothelial cells, is a known predictive marker for cardiovascular events in the general population.[104]

Additional candidate biomarkers for chemotherapy-related endothelial damage include factors involved in endothelial pathobiology, such as inflammation and fibrinolysis. Furthermore, circulating endothelial cells (CECs) in blood may be a sensitive biomarker. CECs, normally present in the circulation in low quantities,[105] have consistently increased levels in cardiovascular, malignant and inflammatory diseases.[106] Recently, elevated CECs and soluble inter-cellular adhesion molecule-1 levels (sICAM-1) were reported in testicular cancer survivors.[20] It can be concluded that only limited and scattered data exist on vascular biomarkers for chemotherapy-related endothelial damage in testicular cancer patients.

We therefore performed a prospective study in testicular cancer patients to investigate changes in a comprehensive panel of biomarkers covering different aspects of vascular pathobiology. This panel includes CECs, plasma markers for endothelial activation, inflammation and pro-thrombotic state and carotid IMT. By measuring levels before, during, 1 month and 1 year after start of cisplatin-based chemotherapy we explored changes in kinetics of these biomarkers. Moreover, associations with clinical cardiovascular events and changes in IMT, a putative early marker of atherosclerosis, were investigated.

PATIENTS AND METHODS

Patients

Eligible for the study were patients with metastatic testicular cancer, 18-50 years old, receiving first line cisplatin-based chemotherapy at the University Medical Center Groningen, the Netherlands. Exclusion criteria were previous chemo- or radiotherapy, presence of CVD, use of

erythropoietin and glomerular filtration rate <60 mL/minute. The local ethics committee approved the study, and written informed consent was obtained from all participants.
Patients received, depending on their International Germ Cell Cancer Collaborative Group (IGCCCG) prognosis group, 3 or 4 3-weekly BEP-courses [bleomycin (30 USP, days 2, 8 and 15), etoposide (100 mg/m^2, days 1-5) and cisplatin (20 mg/m^2, days 1-5)]. During the first 6 days patients were hydrated with 4 L NaCl 0.9%/day and received daily anti-emetic therapy (dexamethasone, ondansetron).

Timing and contents of study-related investigations
Blood samples were drawn before chemotherapy administration at day 1, 8 and 15 [c1d1 (= baseline), c1d8, c1d15, c2d1, etc.] of each BEP-course, 1 month after completion of 3 or 4 BEP-courses and 1 year after start of treatment.
A comprehensive panel of biomarkers was composed of factors involved in different aspects of vascular pathobiology. These included CECs and plasma markers for endothelial activation [vWF, sICAM-1, soluble vascular adhesion molecule (sVCAM-1)], inflammation [fibrinogen, high-sensitivity C-reactive protein (hsCRP)] and fibrinolysis [plasminogen activator inhibitor type 1 (PAI-1), tissue-type plasminogen activator (tPA)]. Carotid IMT was determined as structural vascular biomarker.
CECs, vWF, fibrinogen, PAI-1 and tPA were determined at all visits; hsCRP, sICAM-1 and sVCAM-1 on day 1 and 8 of the first 3 courses, and at both follow-up visits. Patients who developed refractory disease or a relapse were excluded from that moment on.

Biomarkers
CECs
Blood for CECs was collected after discarding the first tube, in CellSave Preservative Tubes (Veridex LLC, Raritan, NJ). Samples were kept at room temperature, shipped overnight the Veridex laboratory (Enschede, the Netherlands), and processed within 96 hours with the CellTracks® AutoPrep® System using the CellSearch® Circulating Endothelial Cell Kit.[105] This system immunomagnetically enriches CECs from 4 mL blood using CD146 conjugated ferrofluids. Enriched cells are stained with the nuclear dye 4,6-diamidino-2- phenylindole (DAPI), phycoerythrin (PE)-conjugated CD105, and allophycocyanin (APC)-conjugated CD45. Next, by placing the sample cartridge on the CellTracks® Analyzer II System fluorescence images for DAPI, PE and APC are acquired. CD146+, DAPI+ objects are classified as CECs when they have the morphological appearance of cells that express CD146, DAPI, CD105 and lack CD45.[105]
For reference value, CECs were determined in a random selection of age-matched healthy males (n=72).

Endothelial activation
vWF (reference values 50-150%) was measured in citrate plasma, as described earlier.8,21 The soluble adhesion molecules sVCAM-1 and sICAM-1 were assessed in EDTA-plasma using a multiplex assay for Luminex technology (Fluorkine MAP® Human Adhesion Molecule Base Kit, R&D Systems Europe Ltd., Abingdon, UK).

Systemic inflammation

The inflammatory proteins fibrinogen (reference values 1.7-4.0 g/L) and hsCRP (reference values <3.0 mg/L) were determined in citrate and EDTA-plasma, respectively, as described earlier.[21]

Pro-thrombotic state

The pro-thrombotic PAI-1 (reference values 3-43 µg/L) and the fibrinolytic tPA (reference values 1.5-10 µg/L) were in citrate plasma measured by ELISA.[8] PAI-1/tPA-ratio was calculated; increases indicate enhanced pro-thrombotic activity.

Vascular structure: IMT

IMT of the right common carotid artery was measured as described earlier8 at baseline and both follow-up visits. An IMT ≥0.02 mm higher than baseline was regarded an increase. To exclude high intra- and inter-assessment variability, intra-individual IMT-measurements with a standard deviation >0.1 mm were excluded.

Cardiovascular Risk Factors

Risk factors were estimated at baseline and follow-up visits. Hypertension was defined as blood pressure (BP) systolic ≥150 mmHg and/or diastolic ≥95 mmHg, or use of anti-hypertensive medication. Body Mass Index (BMI) ≥27.8 kg/m^2 was defined as obesity; fasting total cholesterol ≥6.5 mmol/L and/or use of lipid-lowering medication as dyslipidemia; and diabetes mellitus as fasting glucose >7.0 mmol/L and/or use of glucose-lowering medication.

Statistics

Statistical analyses were performed in SPSS software package version 16.0 (SPSS Inc., Chicago, IL). Non-normally distributed data were represented as median (range). For comparisons between groups the non-parametric Mann-Whitney U test or Kruskal-Wallis test was applied, as appropriate. The Wilcoxon's signed rank test was used for intra-individual changes. Correlations were calculated with Spearman's correlation (r_s). To assess cumulative changes in time, area under the curve (AUC) values for biomarkers at sequential sampling points were calculated in the software package Graphpad Prism version 4.03 (Graphpad Software Inc., La Jolla, Ca). AUCs were calculated for absolute changes in biomarkers during the whole chemotherapy period up to one month post-chemotherapy (all time-points – day 1). Two-sided P-values ≤0.05 were considered significant.

RESULTS

Patients

Between May 2006 - June 2009, 41 patients were included (Fig 1).

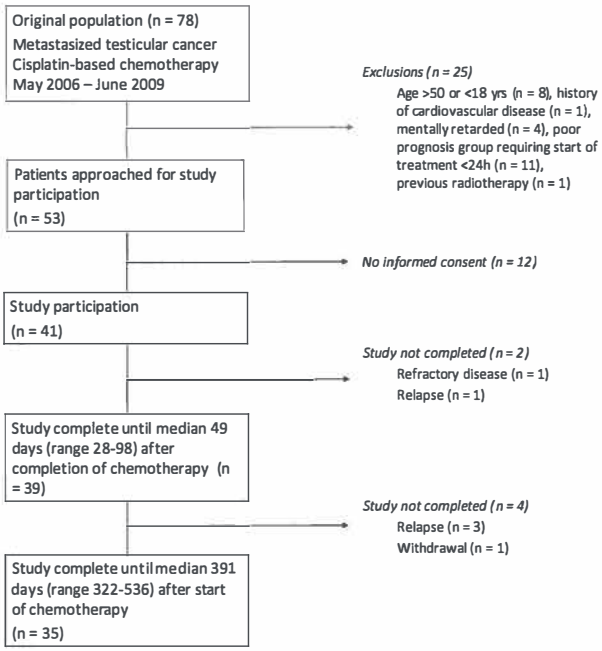


Figure 1. Patient accrual and timing of study-related investigations.

Table 1 summarizes patient characteristics and baseline cardiovascular risk factors. During follow-up, 39 patients were evaluable for the first visit at a median of 49 days (range 28-98), and 35 for the second visit at a median of 391 days (range 322-536) from baseline. Reasons for not completing all visits are provided in Figure 1.

		Median (range)	Number(%)
Number of patients			41
Age at start of treatment, years		31 (18-46)	
Diagnosis			
	Non-seminoma		35 (85.4)
	Seminoma		6 (14.6)
IGCCCG prognosis category			
	Good		33 (80.5)
	Intermediate		7 (17.1)
	Poor		1 (2.4)
Treatment regimen			
	3 cycles BEP		29 (70.7)
	4 cycles BEP		11 (26.8)
	4 cycles EP		1 (2.4)
Granulocyte stimulating factor during treatment			
	Yes		4 (9.8)
	No		37 (90.2)

Table 1 - continued

Red blood cell transfusion during treatment			
	Yes		5 (12.2)
	No		36 (87.8)
Cardiovascular risk factors pre-treatment			
	Hypertension *		0
	Dyslipidemia †		1 (2.4)
	Obesity ‡		9 (23.1)
	Diabetes Mellitus §		0
	Smoking status	*Active smoker*	10 (24.4)
		Ex-smoker	22 (53.7)
		Lifelong non-smoker	9 (22.0)

Table 1. Patient- and treatment-related characteristics. Abbreviations: International Germ Cell Cancer Collaborative Group (IGCCCG); bleomycin-etoposide-cisplatin chemotherapy (BEP).
(*) Hypertension: systolic blood pressure ≥ 150 mmHg / diastolic blood pressure ≥ 95 mmHg, and/or use of anti-hypertensive medication; (†) Dyslipidemia: fasting total cholesterol > 6.50 mmol/L and / or use of lipid-lowering medication; (‡) Obesity: body moss index ≥ 27.8 kg/m2; (§) Diabetes mellitus: fasting glucose > 7.0 mmol/L and/or use of glucose-lowering medication

Biomarkers

Median levels of the vascular biomarker panel at baseline and both follow-up visits are summarized in Table 2. Supplementary Table I shows results for CECs, vWF and IMT in individual patients.

	Baseline		One month after completion of BEP		One year after Start of BEP		Correlation with CECs*			
	Median	Range	Median	Range	Median	Range	Baseline	1 mo	1 yr	AUC
CECs (cells/4 mL)	25	6-75	58 †	23-316	14 †‡	4-41				
vWF (%)	100	42-297	130 †	56-249	115 †‡	49-218	0.12	0.34 §	0.02	0.63 ¶
sVCAM-1 (ng/mL)	552	279-913	583 †	335-1173	601 †	296-1088	0.46 ¶	0. 07	-0.23	0.13
sICAM-1 (ng/mL)	242	116-476	318 †	119-867	285 †	136-581	0.05	0.29	-0.14	0.40 §
Fibrinogen (g/L)	2.8	1.5-5.8	3.0	1.8-5.1	2.5 ‡	1.8-4.2	-0.13	-0.22	0.26	0.16
hsCRP (mg/L)	2.0	0.2-87.1	2.2	0.4-29.1	1.5	0.2-14.3	-0.02	-0.19	0.41 §	0.13
PAI-1 (µg/L)	27	4-98	32	6-126	23 †	8-55	-0.36§	-0.14	0.13	-0.22
tPA (µg/L)	7.7	3.9-12.4	7.8	3.7-28	8.7 †	4.2-17.0	-0.46¶	-0.24	0.07	-0.07
PAI-1/tPA ratio	4.2	1.0-11.7	4.2	0.8-12.6	2.8 †‡	0.1-5.9	-0.25	-0.07	0.00	-0.23

Table 2 – continued

	Baseline		One month after completion of BEP		One year after Start of BEP		Correlation with CECs*			
	Median	Range	Median	Range	Median	Range	Baseline	1 mo	1 yr	AUC
IMT mean (mm)	0.53	0.44-0.82	0.52	0.43-0.76	0.53	0.39-0.79	-0.38§	-0.07	-0.21	0.27 *
Systolic BP (mmHg)	130	120-150	121	100-160	120 †‡	100-150				
Diastolic BP (mmHg)	81	67-100	80	60-100	75 †	60-100				
Cholesterol (mmol/L)	4.8	2.7-7.5	5.1	3.4-7.1	5.2	1.4-6.8				
BMI (kg/m^2)	25.2	20.3-35.8	25.8	21.5-34.3	25.9 †	20.8-33.8				

Table 2. Vascular biomarkers, Intima Media Thickness and cardiovascular risk factors at baseline, one month after completion and one year after start of cisplatin-based chemotherapy for testicular cancer, and correlation coefficients with (changes) in CECs. Abbreviations: bleomycin-etoposide-cisplatin chemotherapy (BEP); circulating endothelial cells (CECs); von Willebrand Factor (vWF); soluble inter cellular adhesion molecule (sICAM-1); soluble vascular cellular adhesion molecule (sVCAM-1); plasminogen activator inhibitor type 1 (PAI-1); tissue-type plasminogen activator (tPA); high-sensitivity c-reactive protein (hsCRP); intima media thickness of the common carotid artery (IMT); blood pressure (BP); body mass index (BMI); area under the curve (AUC).
(*) Spearman correlation coefficients for correlation between CECs and markers/IMT at baseline, one month after completion and one year after start of treatment, and for AUC for changes in time throughout treatment [delta IMT (baseline-one month; baseline-one year)]; (†)P <0 .01 compared to baseline, Wilcoxon signed rank test; (‡) P <0 .01 compared to one month after completion of treatment, Wilcoxon signed rank test; (§) significance for correlation P < .05; (¶) Significance for correlation P < .01.

CECs

Baseline CECs were independent of age (r_s = -0.25; P=0.15), IGCCCG prognosis group [good: median 22 CECs/4 mL (range 6-75), intermediate: median 31 CECs/4 mL (range 14-54), poor: 25 CECs/4 mL (n=1); P=0.66], or smoking status [active smokers: median 23.5 CECs/4 mL (range 7-74), lifelong non-smokers: median 27 CECs/4 mL (range 6-75), ex-smokers: median 22 CECs/4 mL (range 6-54); P=0.58].

Compared to baseline, CECs significantly increased over consecutive BEP-courses (Fig 2A), with a median 5.6-fold maximal increase (range 1.2-34.6). The median intra-individual maximal CECs peaked on c3d8 (range c1d8-1 month post-chemotherapy). CECs were persistently higher than baseline 1 month post-chemotherapy. One year post-chemotherapy CECs declined compared to baseline, and equaled healthy males (Fig 3A).

Excluding data from CECs in patients receiving granulocyte-colony stimulating factor (n=4) and red blood cell transfusions (n=5) did not change the results. CEC-levels were not different between patients who received 3 or 4 BEP-courses (data not shown).

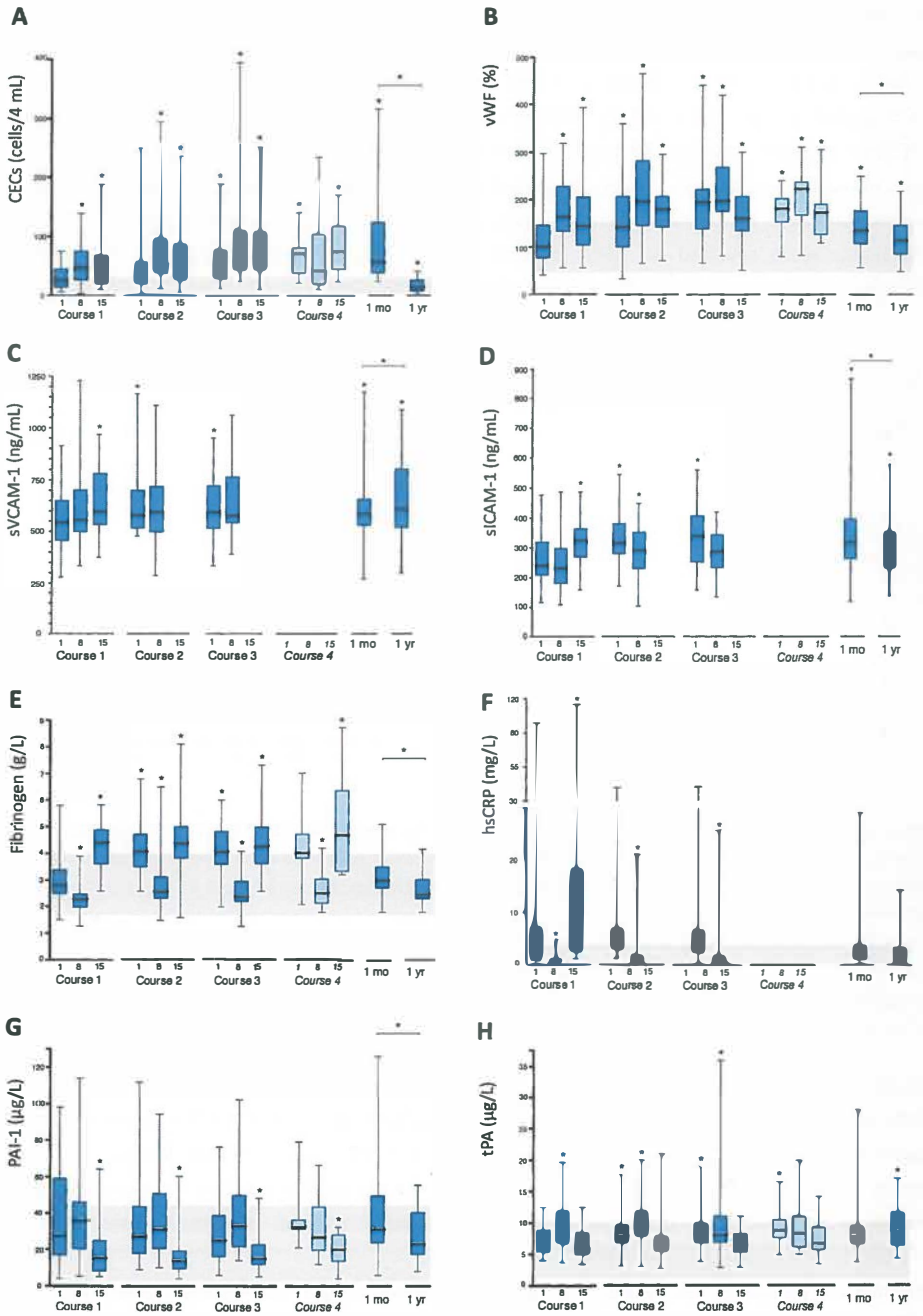


Figure 2. Circulating endothelial cells (CECs, A), von Willebrand Factor (vWF, B), soluble vascular adhesion molecule 1 (sVCAM-1, C), soluble intra-cellular adhesion molecule 1 (sICAM-1, D), fibrinogen (E), high-sensitivity C-reactive protein (hsCRP, F), plasminogen activator Inhibitor 1 (PAI-1, G) and tissue-type plasminogen activator (tPA, H) before, during and after completion of cisplatin-based chemotherapy for testicular cancer. The sample at c1d1 is drawn before initiation of chemotherapy. The box-and-whisker plots show the 10th, 25th, 50th, 75th and 90th percentile. Levels of markers in n = 11 patients during the 4th BEP-course are indicated in light blue.

(*) P<0.05 compared to baseline value or indicated time-point (Wilcoxon signed rank test); the grey areas comprise reference values. References values for CECs (1-20 CECs/4 mL) are based on a study in 249 healthy individuals.[105]

Markers for endothelial activation

Baseline endothelial activation markers did not differ between IGCCCG-groups. Baseline sICAM-1, but not vWF or sVCAM-1, levels were higher in smokers [active smokers: median 348.9 pg/mL (range 116.9-438.8), lifelong non-smokers: median 215.9 pg/mL (range 137.0-475.5) ex-smokers: median 225.1 pg/mL (range 200.7-423.8); P=0.02].

vWF, sVCAM-1 and sICAM-1 levels increased during BEP-courses (Fig 2B, 2C, 2D). The median maximal increase in vWF-levels was 2.2-fold (range 1.0-4.6), median intra-individual maximal vWF-levels were reached on c3d1 (median; range baseline-c4d8). Compared to baseline, endothelial activation markers were higher at both follow-up visits.

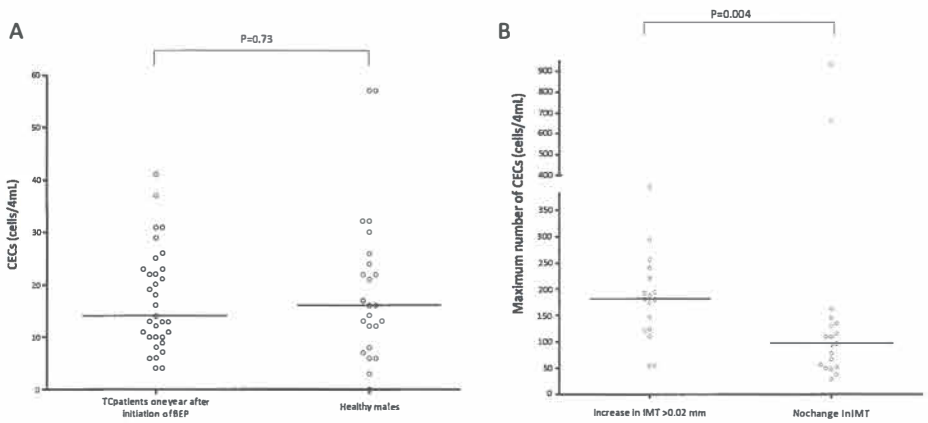


Figure 3A. Levels of CD146+, CD105+, DAPI+, CD45- circulating endothelial cells (CECs) in testicular cancer (TC) patients [median age 32 years (range 19-47); median CECs 14 cells/4 mL (range 4-41)] one year after start of BEP-chemotherapy, compared to healthy age-matched males [median age 33 years (range 21-47); median CECs 16 cells/4 mL (range 0-57)].

Figure 3B. Maximal levels of CECs in testicular cancer patients with an increase in IMT of > 0.02 mm compared to baseline [median 180 cells/4 mL (range 54-294)], versus patients without an increase in IMT [median 103 cells/4 mL (range 29-933); P=0 .004]. Lines indicates median level of CECs/4 mL in each group; P-value are calculated with the Mann-Whitney U test.

Markers for inflammation

Patients in the IGCCCG intermediate prognosis group had higher baseline fibrinogen and hsCRP levels [fibrinogen/hsCRP: good prognosis: median 2.7 g/L (range 1.5-5.4)/ median 1.5 mg/L (range 0.2-87.1); intermediate prognosis: median 4.3 g/L (range 3.1-5.8)/ median 18.6 mg/L (range 0.7-39.6); poor prognosis: median 3.7 g/L / median 5.4 mg/L (n=1 patient); P=0.003/0.03]. Levels were not different in smokers.

Inflammatory markers decreased during the first week of each BEP-course, which concurred with administration of steroids as anti-emetic (Fig 2E, 2F). Post-chemotherapy, fibrinogen and hsCRP levels were not different from baseline.

Markers for pro-thrombotic state

Baseline levels of PAI-1, tPA or PAI-1/tPA-ratio did not differ between IGCCCG prognosis groups or between smokers and non-smokers.

Levels of PAI-1 and tPA changed during the BEP-courses (Fig 2G, 2H). One year post-chemotherapy, PAI-1/tPA-ratio was lower than baseline (Table 2), indicating a less pro-thrombotic state.

Marker for vascular structure

At baseline, IMT was related to age (r_s=0.35; P=0.04) and BMI (r_s=0.52; P=0.002), but not to BP or cholesterol. These correlations were absent during follow-up. Median IMT for the group as a whole did not change over time (Table 2).

Relations between CECs and plasma biomarkers

Table 2 summarizes correlations between CECs and other biomarkers. Baseline vWF and CECs were not related, whereas they moderately correlated 1 month post-chemotherapy. One year post-chemotherapy, CECs correlated with hsCRP. When all markers were taken into account at each time-point, CECs correlated with vWF (r_s=0.33; P <0.0001) and fibrinogen (r_s=0.15; P=0.001),

During the first 3 BEP-courses, changes in CECs and vWF followed a similar pattern (Fig 2A, 2B, supplementary Fig I). Median percentual levels of CECs and vWF at each subsequent time-point strongly correlated (r_s=0.76; P=0.005).

Cumulative changes in CECs during all BEP-courses up to 1 month post-chemotherapy correlated with vWF and sICAM-1 (r_s for AUC=0.63; P=0.001 and r_s=0.40; P=0.03; Table 2). Cumulative changes in tPA and PAI-1 (r_s for AUC=0.37; P=0.02) and fibrinogen and hsCRP (r_s for AUC=0.42; P=0.007) throughout all BEP-courses up to 1 month post-chemotherapy correlated.

Relations between increases in IMT and CECs and plasma biomarkers

During follow-up visits, IMT increased in 17 patients (41.5%, Supplementary Table I). Patients with an increase in IMT had higher maximal CECs (Fig 3B) and larger cumulative changes in CECs. Patients with an increase in IMT had a median CEC-AUC of 7,359 (range 1,994-30,083), while the others had a median CEC-AUC of 5,299 (range 1,107-14,322; P =0.044). Patient- or treatment characteristics, cardiovascular risk factors and (changes in) other biomarkers were not related to increases in IMT.

Cardiovascular events and risk factors

One patient presented with pulmonary embolism prior to chemotherapy. Five out of 41 patients (12.2%) developed a cardiovascular event during or after completion of chemotherapy (Table 3). Overall, median percentage changes in CECs were significantly larger in the patients with a vascular event since start of chemotherapy, compared to the other patients (Fig 3C). In three of them CECs did not increase before but directly after the event. Patterns of changes in the other biomarkers were not distinctive in patients with vascular events.

Compared to baseline, median systolic and diastolic BP and BMI were higher 1 year post-chemotherapy (Table 2). Patient- or treatment characteristics, (changes in) vascular biomarkers or IMT were not related to changes in risk factors.

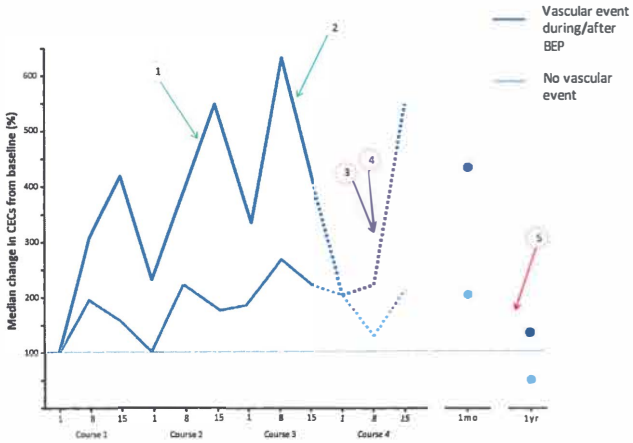


Figure 3C. Median percentage changes in CD146+, CD105+, DAPI+, CD45- Circulating Endothelial Cells (CECs) in patients with (dark line) and without cardiovascular events (light line) during or after BEP-chemotherapy, compared to baseline (= 100%). Median changes are larger in patients with cardiovascular events during or after completion of chemotherapy (P=0.001, Mann Whitney U test). Dotted lines indicate changes in patients (n = 11) during 4th course. Timing of the events is indicated with an arrow, numbers correspond with patients in Table 3, where details concerning the event are given. Green arrows indicate venous thrombo-embolic events, red arterial thrombo-embolic events.

Patient	Age	CVD risk factors	Event	Timing	Outcome
1	35	None	Pulmonary embolism	During second course	Recovery without residual impairments
2	27	None	Pulmonary embolism (asymptomatic)	During third course; accidental finding on CT-scan	Recovery without residual impairments
3	43	None	Transient ischemic attack and renal infarction	During fourth course	Recovery without residual impairments
4	45	Smoking, family history for CVD	Ischemic cerebrovascular accident	During fourth course	Died from progressive disease eight months after start of chemotherapy
5	28	Smoking, family history for CVD	Ischemic cerebrovascular accident	12 months after start of treatment	Recovery without residual impairments

Table 3. Characteristics of testicular cancer patients with vascular events during or after completion of BEP-chemotherapy. Patient numbers correspond with the numbers indicated in Supplementary Fig I. Abbreviations; cardiovascular disease (CVD), computed tomography (CT) scan

DISCUSSION

In this prospective study in metastatic testicular cancer patients we evaluated changes in a comprehensive panel of factors involved in vascular pathobiology. Within this panel, CECs appeared of interest as candidate biomarker for early chemotherapy-related endothelial damage. Changes in CECs correlated with changes in the endothelial activation markers vWF and sICAM-1, and were larger in patients with increases in their carotid IMT and in those with

early vascular events. Another promising marker from this panel of biomarkers was vWF, with increases in plasma vWF-levels during chemotherapy that persisted up to one year after treatment.

CECs are considered promising real-time cellular biomarkers for different vascular damage conditions, as these cells are considered to have detached from damaged vessel walls.106 This opinion is underscored by the finding that CECs are generally low in healthy individuals, but raised in inflammatory, malignant and cardiovascular diseases.[105-107]

However, as technologies and definitions currently used to measure CECs vary, it is unlikely that the same events are scored in the studies reported until now.[106,108] Here we used a standardized and validated method that defines CECs as cells that express both CD146 and CD105, lack the pan-leukocyte marker CD45 and have a nucleus present. This automated method selects cells with typical endothelial cell gene expression,[109] that peak upon vascular damage[110] and correlate with vascular damage biomarkers.[107] Increases in this cell population occur during chemotherapy, i.e. docetaxel for metastatic prostate cancer.[111]

Although we found no relation between baseline CECs and IGCCCG-prognosis group, the CEC-level can be influenced by neovascularization related to the metastatic malignancy. This is underscored by the finding that CEC-levels were lower than baseline at one year post-chemotherapy, and comparable to healthy age-matched males. The cumulative increase in CECs that we observed during chemotherapy can be a resultant of several factors. Firstly, CECs will likely be released from tumor vessels in response to effective treatment. As most patients had normalization of tumor markers after one chemotherapy course, at that early time-point the tumor load was already substantially decreased. Therefore, other factors will also play a role, like chemotherapy-induced damage that causes shedding of endothelial cells from systemic vasculature. This is apparent from a recent study reporting rises in CECs in cancer patients receiving adjuvant chemotherapy.[112] Furthermore, repeated venipunctures and the vacuum blood drawing may increase CECs. To our knowledge it is unknown whether drugs used to alleviate side effects of chemotherapy, such as steroids, induce changes in CECs.

Another promising marker in our biomarker panel is the endothelial activation marker vWF. Plasma vWF-levels increased during BEP-courses, in line with an earlier study.[8] The patterns of rises in CECs and vWF were similar in our study, and cumulative changes in CECs, vWF and sICAM-1 correlated. Similar correlations between CECs and vWF have been reported in several other vascular damage conditions,[113,114] supporting the hypothesis that changes in these biomarkers reflect chemotherapy-related endothelial damage. Additionally, levels of vWF and soluble adhesion molecules remained higher than baseline up to 1 year post-treatment, suggesting persisting endothelial activation. As endothelial activation is a first step in vascular pathophysiology, this can progress to accelerated atherosclerosis.[30] Within our panel of vascular biomarkers we also observed changes in markers for inflammation and pro-thrombotic state during BEP-chemotherapy, suggesting that these phenomena add to the development of vascular complications.

Changes in IMT are used as intermediate endpoint in cardiovascular intervention trials.[115-117] Earlier, we observed a 0.02 mm increase in IMT ten weeks after 4 BEP-courses.8 Absence of change in median IMT in the current study is likely due to the lower cumulative cisplatin dose administered in the majority of our patients (73% received 3 BEP-courses). Although we did not find differences in vascular biomarkers between patients treated with 3 or 4 BEP-courses, it is plausible that a higher cumulative dosis of chemotherapy induces more vascular damage.

In this study increases in IMT ≥0.02 mm, as observed in 17 patients, related to higher maximal CEC-levels and larger cumulative changes in CECs. This suggests that patients with larger increases in both CECs and IMT represent a subgroup with augmented vascular damage, which may progress to accelerated atherosclerosis in the long term.

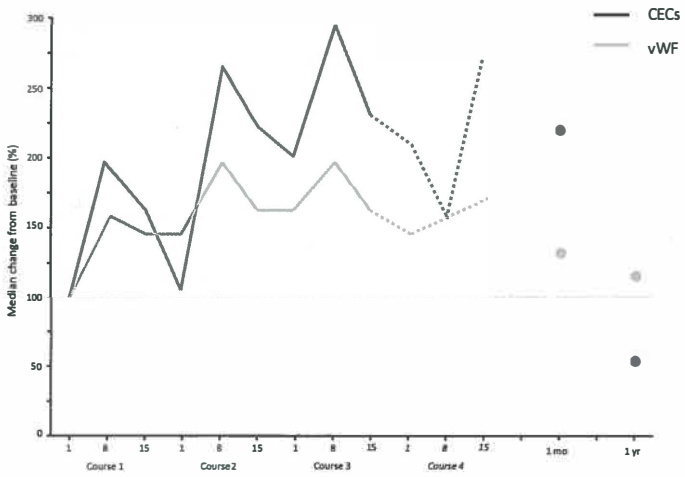
There are two known peaks in occurrence of vascular complications in testicular cancer patients; acute events manifest during or shortly after chemotherapy,[9,102] whereas chronic consequences arise up to decades post-treatment.[12-14,16,103] In the current study, changes in CECs, but not in other biomarkers, were larger in patients with early vascular events. Large increases in CECs may point to patients in whom intervention to prevent progression towards endothelial dysfunction and CVD is indicated. The relatively large inter-individual variation in CEC-levels which we observed still hampers the definition of an optimal cut-off level from these data. It is known from studies in the general population that IMT-measurement has a relatively large inter-measurement varation,[118] implying that changes in IMT in individual patients over a short follow-up period is not suitable as predictive vascular biomarker.

In this study in a comprehensive panel of vascular biomarkers, changes in levels of CECs and vWF as well as carotid IMT may point to testicular cancer patients with augmented endothelial damage. These biomarkers should be included in studies addressing the cisplatin chemotherapy-related damage phenotype in testicular cancer patients, consisting of accelerated atherosclerosis and cardiovascular morbidity. In extended follow-up studies this will reveal the predictive value of early rises in CECs and persistent endothelial activation for late CVD in this patient cohort. Eventually, such a marker panel should identify patients in whom active intervention to alleviate or prevent chemotherapy-related CVD is indicated.

Acknowledgments

We thank Anne van Gessel, Annet Possel-Nicolai, Margreet Teune-Weesjes, Marianne Bruin and Wietze Kuipers for measurements of carotid IMT and Sandra Geerards for measurements of tPA/PAI-1. This work was supported by Veridex LLC and with grant 2009-4365 from the Dutch Cancer Society.

SUPPLEMENTARY FIGURES AND TABLES



Supplementary Figure I. Median relative changes in CD146+, CD105+, DAPI+, CD45- circulating endothelial cells (CECs) and von Willebrand Factor (vWF) during, one month after completion and one year after start of BEP-chemotherapy, compared to baseline (= 100%). The dark line represents median changes in CECs, the light line vWF. Changes in CECs and vWF in n = 11 patients during the 4th course are indicated as a dotted line.

Supplementary Table I. Age, disease characteristics [disease stage, International Germ Cell Cancer Collaborative Group (IGCCCG) prognosis group] and occurrence of cardiovascular events during or after BEP-chemotherapy, individual levels of CD146+, CD105+, DAPI+, CD45- circulating endothelial cells (CECs), levels of von Willebrand Factor (vWF) and intima media thickness of the common carotid artery (IMT) at baseline, maximum level throughout the whole study period, timing of maximum level, levels at one month after completion and one year after start of cisplatin-based chemotherapy. (nd = not done)

Age at baseline	Disease stage	IGCCCG	Vascular event during/after BEP	CECs(cells/4mL)					vWF (%)					IMT(mm)			
				Baseline	Maximum level	Timing of max level	1 mo	1 yr	Baseline	Maximum level	Timing of max level	1 mo	1yr	Baseline	1 mo	1 yr	Increase >0.02 mm
18	4	Good	no	43	218	c2d8	48	7	53	195	c3d8	70	69	n.d.	0.61	0.49	n.d.
19	2	Good	no	17	57	c1d8	32	31	62	230	c3d8	56	69	0.51	0.51	0.53	no
22	2	Good	no	60	180	c2d8	147	26	86	192	c3d15	135	102	0.47	0.51	0.56	yes
22	3	Intermediate	no	27	933	c3d15	66	9	122	278	c2d8	159	131	0.49	0.46	0.45	no
23	2	Good	no	16	54	c2d8	34	10	42	89	c3d8	84	60	0.55	0.66	0.62	yes
23	3	Intermediate	no	25	129	c4d15	40	16	50	175	c1d8	75	49	0.53	0.43	0.47	no
25	2	Good	no	29	54	c2d8	44	6	101	464	c3d8	122	155	0.47	0.45	0.50	yes
26	2	Intermediate	no	34	47	c3d8	26	n.d.	159	263	c3d8	146	135	0.67	n.d.	n.d.	n.d.
26	4	Good	no	27	94	c2d8	80	41	98	187	c3d8	n.d.	114	0.47	0.47	n.d.	no
26	2	Good	no	42	145	c3d8	78	37	121	419	c3d8	195	164	0.47	0.46	0.44	no
27	4	Intermediate	no	54	320	c3d8	n.d.	n.d.	156	239	c4d8	n.d.	n.d.	0.47	n.d.	n.d.	n.d.
27	4	Poor	Pulm.embolism	25	221	c2d15	23	12	99	224	c4d8	148	96	0.48	0.57	0.53	yes
27	2	Good	no	38	135	c2d15	95	20	51	131	c3d8	n.d.	61	0.44	0.44	0.47	yes
27	2	Good	no	7	111	c3d8	121	23	111	211	c3d1	150	121	0.66	0.57	0.53	no
28	2	Good	CVA	8	175	c3d8	46	11	137	284	c3d8	211	169	0.63	0.72	0.70	yes
28	2	Good	no	22	50	c2d8	44	22	92	190	c2d8	107	101	0.66	n.d.	0.66	no
29	2	Good	no	42	48	c4d1	71	21	72	207	c2d15	124	107	0.51	0.53	0.55	yes
29	2	Intermediate	no	n.a.	124	c1d8	29	8	297	297	c4d8	137	127	0.59	0.53	0.56	no
30	3	good	no	25	109	c1d8	38	29	99	229	c4d8	123	115	0.54	0.47	0.47	No
31	2	good	no	38	663	c3d8	64	11	93	250	c3d1	160	122	0.73	0.69	0.58	No
31	2	good	no	57	66	c3d15	51	4	96	202	c3d8	138	108	0.67	0.59	0.57	No
33	2	good	no	n.d.	52	c1d8	52	10	109	297	c2d8	124	105	0.45	0.56	0.51	Yes
33	2	good	no	21	147	c2d8	35	n.d.	n.d.	374	c3d8	217	n.d.	0.56	0.48	n.d.	No

Supplementary Table I – continued

Age at baseline	Disease stage	IGCCCG	Vascular event during/after BEP	CECs (cells/4mL)					vWF (%)					IMT (mm)			
				Baseline	Maximum level	Timing of max level	1 mo	1 yr	Baseline	Maximum level	Timing of max level	1 mo	1 yr	Baseline	1 mo	1 yr	Increase >0.02 mm
34	2	good	no	44	294	c2d8	45	n.d.	256	360	c2d1	158	n.d.	0.51	0.63	n.d.	Yes
34	2	good	no	n.d.	162	one mo	162	14	162	369	c3d8	196	156	0.53	0.49	0.39	No
34	2	good	no	10	29	one mo	29	13	50	107	c2d8	63	76	0.46	0.45	0.42	No
35	2	good	Pulm.embolism	n.d.	241	c2d8	126	n.d.	76	304	c2d8	122	123	0.55	0.53	0.60	Yes
35	2	good	no	6	96	c3d8	n.d.	10	80	209	c2d15	108	76	0.63	0.52	0.59	No
36	4	intermediate	no	51	256	one mo	256	23	115	363	c1d15	88	91	0.53	0.56	0.54	Yes
36	2	good	no	15	192	one mo	192	n.d.	149	292	c3d15	174	n.d.	0.58	0.63	n.d.	Yes
37	3	good	no	14	109	c3d15	82	n.d.	147	316	c3d8	219	184	0.54	0.45	0.53	No
38	4	good	no	30	38	c1d15	34	4	78	126	c3d1	62	73	0.79	n.d.	0.79	No
38	2	good	no	74	395	c3d8	168	25	100	150	c1d8	n.d.	152	0.54	0.57	0.86	Yes
41	4	good	no	28	115	c3d8	116	13	161	440	c3d1	249	218	0.46	0.45	0.51	Yes
41	2	good	no	22	188	c1d15	40	6	81	210	c3d8	107	101	0.76	0.76	0.74	No
42	2	good	no	75	316	one mo	316	18	148	394	c3d1	213	146	n.d.	n.d.	n.d.	n.d.
42	2	good	no	18	121	c2d8	n.a.	22	n.a.	272	c3d1	120	119	0.56	0.66	n.a.	Yes
43	2	intermediate	CVA	14	195	c3d8	42	19	145	311	c4d15	179	149	0.75	0.75	0.54	No
43	2	good	no	21	77	c4d15	144	13	160	318	c3d8	119	183	0.68	0.60	0.79	Yes
45	2	good	CVA	10	69	one mo	69	n.d.	146	262	c3d15	200	n.d.	0.82	n.d.	n.d.	n.d.
46	2	good	no	6	181	c3d1	132	31	72	253	c3d8	109	61	0.45	0.44	0.55	Yes

Evaluation of sub-acute changes in cardiac function after cisplatin-based combination chemotherapy for testicular cancer

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Chapter 4

ABSTRACT

Long-term cardiovascular morbidity is increasingly observed in chemotherapy-treated testicular cancer survivors, but little is known of early sub-clinical changes in cardiac function. We prospectively evaluated cardiac function by echocardiography. Systolic (Wall Motion Score Index) and diastolic [E/A-ratio and Tissue Velocity Imaging (TVI)] parameters, and serum levels of N-Terminal pro-Brain Natriuretic Peptide (NT-proBNP) were assessed before start of chemotherapy and one year later. Echocardiography data were compared with an age-matched group of healthy controls. 42 patients treated with Bleomycin, Etoposide and Cisplatin were evaluated (median age 27 yrs, range 18-50). Systolic function and E/A-ratio did not change, whereas the median TVI decreased (12.0 Vs 10.0 cm/s; p=0.002). Median levels of NT-proBNP increased (5 Vs 18 pmol/L, p=0.034). Compared with controls, TVI before the start of chemotherapy was not significantly different. In conclusion, at a median of 10 months after cisplatin-based treatment for testicular cancer, TVI decreased significantly, indicating a deterioration of diastolic cardiac function. Serum levels of NT-proBNP increased. The prognostic significance of these changes for future cardiovascular morbidity is not clear.

INTRODUCTION

Testicular cancer (TC), the most frequent type of solid malignancy in young male adults, has become a highly curable disease since the late seventies with the introduction of cisplatin-containing chemotherapy regimens. In the growing population of TC survivors, the occurrence of long-term treatment induced organ damage is increasingly recognized as an important cause of morbidity.2 Compared with the general population, long-term TC survivors have a higher incidence of second malignant neoplasms and cardiovascular disease.[7,12-14]

In earlier studies, investigating cardiac morbidity in long-term TC survivors median 7 to 14 years after chemotherapy, we found diastolic dysfunction in 17-33% of patients.[13,36] Sub-clinical signs of vascular toxicity were found in a prospective study in TC patients 10 weeks after cisplatin-based chemotherapy.[8] Still, little is known about (sub-) acute cardiotoxicity in TC patients, and to our knowledge, no studies report on early cardiotoxicity. Furthermore, it is not established which parameters are useful in the early assessment of cardiac damage.

In addition to obtaining insight in the extent and timing of cardiac complications of chemotherapeutic treatment for TC, evaluation of parameters for early (sub-clinical) cardiac dysfunction may enable identification of patients at risk for future cardiovascular events. Echocardiography is a convenient and frequently used method to assess cardiac function, enabling evaluation of both systolic and diastolic function parameters. Biochemical markers can also be used to evaluate cardiac status. N-Terminal pro-Brain Natriuretic Peptide (NT-proBNP) is produced by ventricular cells in response to increased mechanical load and wall stretch. Plasma levels are used as a prognostic indicator in different stages and causes of cardiac disease.[119] However, its role in detecting chemotherapy-induced cardiac morbidity has not been established yet.[120,121]

In this prospective cohort study, we investigated echocardiographic and biochemical changes before and one year after start of cisplatin-containing chemotherapy for disseminated TC.

PATIENTS AND METHODS

Patients

All consecutive patients with disseminated TC, scheduled to receive cisplatin-containing chemotherapy as first line therapy at the University Medical Centre Groningen, the Netherlands, between December 2000 and October 2004 were asked to participate in a study investigating chemotherapy-induced acute cardiovascular toxicity.[8] Exclusion criteria were extra-pulmonary visceral metastases, previous radiotherapy, pre-treatment history of cardiac disease, use of erythropoietin and an age older than 55 years at the start of chemotherapy. The study was approved by the local ethics committee and written informed consent was obtained from all participants.

Following orchidectomy, all patients received three or four three-weekly courses of combination chemotherapy consisting of bleomycin (30 mg on days 2, 8 and 15), etoposide (100 mg/m^2 on days 1 to 5 of each course) and cisplatin (20 mg/m^2 on days 1 to 5 of each course). Patients were admitted to the hospital for hydration with 3 liters NaCL 0.9%/day during the first 6 days of each course. All patients received dexamethason and odansetron as standard anti-emetic therapy.

Reference data were obtained from healthy male siblings of adult childhood cancer survivors, who had participated as control subjects in a cross-sectional study on late cardiovascular

sequelae of treatment for childhood cancer. Out of these healthy male siblings, a control group was selected with a comparable median age as the TC patients. Measurements in the controls were performed under similar circumstances and methods.

Measurements

Measurements were done within one week before start of chemotherapy and approximately one year after completion of treatment.

Echocardiography

Echocardiography was performed by a skilled technician at the same laboratory using conventional equipment (General Electrical VIVID 7 system, 2.5 MHz probe) and consisted of two-dimensional echocardiography, colour flow mapping and, since 2002, tissue velocity imaging.[122] Left ventricular end-diastolic dimension (LVEDD, normal 36-54 mm), left ventricular end-systolic dimension (LVESD, normal 23-40 mm), posterior and septal wall thickness (normal 7-11 mm) were measured on M-mode recordings obtained in the standard left ventricular parasternal long axis view. Parasternal, transverse and longitudinal dimensions of the left atrium were attained.

For analysis of systolic function, the left ventricle was divided into 16 segments. Each segment was visually scored between 1 and 4 (1 = normokinesia, 2 = hypokinesia, 3 = akinesia, 4 = dyskinesia). The Wall Motion Score Index (WMSI) was the mean score for all analysed segments. A WMSI of 1.00 was considered normal.

Diastolic function measurements included mitral valve inflow velocities in early (E) and late (atrial; A) diastole (E/A-ratio, normal > 1.00) and Tissue Velocity Imaging of early diastole (TVI Et). TVI Et was the mean of measurements at the septal, lateral, inferior and anterior mitral annulus (normal > 8.0 cm/s; decreases indicate deterioration of diastolic function). In addition E/E' was calculated from the peak E velocity and the mean TVI Et (normal <15; increases reflect declines in diastolic function; E/E' >15 is considered as diastolic dysfunction). E/E' is currently regarded as a sensitive method for assessing diastolic heart failure.[123]

NT-proBNP

NT-proBNP (lower limit of detection 5.0 pmol/L; normal value < 14.75 pmol/L) was measured in plasma using an immunoassay (Roche Diagnostics, Mannheim, Germany), from samples that were drawn concomitant with the echocardiographic recordings.

Cardiovascular risk factors

Cardiovascular risk factors were estimated before start of chemotherapy. Hypercholesterolemia was defined as a fasting level of cholesterol >6.5 mmol/L; diabetes mellitus as a fasting level of glucose > 7.0 mmol/L. Obesity as Body Mass Index (BMI) >27.8 kg/m^2. Blood pressure was estimated as a single recording on one arm in supine position in a quiet room after a minimal rest period of 10 minutes. Furthermore, an ambulatory blood pressure (BP) device (Spacelab 90207; Spacelabs Inc., Redmond, WA, USA) was used to document BP every 30 minutes during a 24-hour period. The control subjects had a single blood pressure recording. The criteria for hypertension were defined as a mean 24-hour BP >135/85 mmHg, a single BP >145/95 mmHg, and/ or the use of anti-hypertensive medication.

Statistics

Statistical analyses were performed in SPSS inc. version 14. For comparisons the Chi-square test and the non-parametric Mann-Whitney test were used. To calculate changes within a patient the Wilcoxon's signed rank test was used on the paired samples, in those patients where both variables were available. Regressions were calculated with Spearman's correlation. Two-sided p-values ≤0.05 were considered to indicate significant differences.

Post-hoc power analysis demonstrated that, with a power of 80% and a two-sided significance level (alpha) of p <0.05, a 3.3% change in TVI Et could be detected in 19 patients, with a correlation coefficient between two measurements under equal circumstances of ≥ 0.75 and variation-coefficient for TVI Et of 7.1%.[124]

RESULTS

Patients

Between December 2000 and October 2004, 65 TC patients were enrolled in the study.[8] In 54 of these 65 patients an echocardiogram was performed before start of chemotherapy. Twelve out of 54 (22%) patients received no echocardiogram after completion of cisplatin-based chemotherapy, because of the following reasons: death (n=1), progressive disease (n=1) or logistic reasons (data not evaluable N=3; moved out of referral area n=1; not performed n=6).

Therefore, 42 patients underwent an echocardiogram before and within one year after completion of cancer treatment. The baseline characteristics like age, disease status and echocardiographic parameters of the patients (n=12) who did not receive an echocardiogram after treatment were not different from the patients that completed two echocardiographic evaluations (data not shown).

Baseline characteristics of the 42 patients with two echocardiographic evaluations are shown in Table 1. Of the 42 evaluable patients, 12 had pulmonary metastases, 1 underwent thoracotomy for resection of pulmonary lesions after completion of chemotherapy. The control group consisted of 42 healthy males, whose median age was 28 years (Table 2); none of them was known to have co-morbidity.

Echocardiography

Echocardiography pre-treatment

Before start of treatment, one out of 33 patients had a WMSI >1.00 (3.0%). Four out of 41 patients had abnormal wall motion (all local hypokinesia; 9.8%). Three out of 42 had valve dysfunction [pulmonal (n=2) and tricuspid valve insufficiency (n=1); 7.1%].

The E/A-ratio was <1.00 in eight out of 42 patients (19.0%); one out of 22 assessed patients had a TVI Et <8.0 cm/s (4.5%). None of these 22 patients had an E/E' >15. Median values and ranges are summarised in Tables 2 and 3.

Of the controls, one of 42 had had a WMSI >1.00. One out 42 had an E/A-ratio <1.00; one out of 42 had a TVI Et mean <8.00 cm/s. For median values and ranges, see Table 2.

The median WMSI and TVI Et in patients and the controls was not different (p=0.259; p=0.713), whereas the median E/A-ratio was higher in the controls (p<0.0001, Table 2).

Number of patients	42
Age at start of treatment	
Median (range)	27 (18-50) years
Interval between echocardiography	
Median (range)	10 (6-15) months
Diagnosis (n)	
Non-seminoma	40
Extra-gonadal non-seminoma	1
Seminoma	1
Stage of disease stage* (n)	
Stage II	28
Stage III	3
Stage IV	11
Prognosis category¶ (n)	
Good	28
Intermediate	14
Poor	0
Treatment (n)	
3 or 4 x BEP†	41
2 x BEP, 1 x VIP‡	1

Table 1. Characteristics of participating patients with testicular cancer.
(*) Royal Marsden classification; (¶) According to the International Germ Cell Cancer Collaborative Group (IGCCCG); (†) BEP: Bleomycin, Etoposide, Cisplatin, the number of cycles was based on the prognostic classification; (‡) VIP: Etoposide, Ifosfamide, Cisplatin

	Patients		**Controls**		**P-value***
	n†	Median (range)	n	Median (range)	
Age	42	27 (18-50)	42	28 (18-42)	0.91
Systolic blood pressure	30	133 (110-180)	42	115 (98-156)	<0.0001
Diastolic blood pressure	30	80 (50-130)	42	79 (6-96)	0.24
Body Mass Index	30	24 (19-38)	42	25 (19-31)	0.66
TVI Et	22	12.0 (7-19)	42	12.4 (9-15)	0.71
E/A ratio	42	1.38 (0.73-2.84)	42	1.69 (1.02-2.66)	<0.0001
WMSI	33	1.00 (1.00-1.23)	42	1.00 (1.00)	0.26

Table 2. Baseline characteristics and cardiac parameters of testicular cancer patients and healthy control subjects
(*) Mann-Whitney test; (†) Number of patients in which the respective parameters were available

Echocardiography post treatment

Median 10 months after completion of treatment, four of 28 patients had a WMSI >1.00 (14.3%). Seven out of 40 patients had wall motion abnormalities (17.5%, local and/or diffuse hypo- and akinesia). The E/A-ratio was <1.00 in seven out of 41 patients (17.1%); two out of 25 assessed patients had a TVI Et <8 cm/s (8.0%). None of the 25 patients had an E/E' >15.

Changes in echocardiography pre and post treatment

Cardiac dimensions and the median WMSI did not change during the year after treatment (Table 3). The percentage of patients with wall motion abnormalities did not change (9.8 Vs 17.5%; p=0.309), whereas the number of patients with a WMSI >1.00 increased (p=0.01). The seven patients with post-treatment wall motion abnormalities included the four patients with pre-treatment abnormalities; all of them had deterioration of wall motion abnormalities. Two

of them developed pulmonary embolisms during treatment.[5] Three patients newly developed wall motion abnormalities one year after treatment; one had had a myocardial infarction during chemotherapy.

Paired observations of the TVI Et were available in 19 TC patients. The median TVI Et decreased significantly after treatment (p=0.002; Figure 1); the median E/A-ratio did not change (see Table 3). In addition, the median E/E' increased (p=0.002; Table 3).

Age correlated with pre-treatment E/A-ratio (R=-0.50; p=0.001), but not with pre-treatment TVI Et (R=-0.27; p=0.219) or delta TVI Et (difference pre- and post treatment TVI Et; R=0.21; p=0.382).

	Before		After		P-value *
	n†	Median (Range)	n	Median (Range)	
SYSTOLIC PARAMETERS					
WMSI	33	1.00 (1.00-1.23)	28	1.00 (0.81-1.44)	0.273
STRUCTURAL DIMENSIONS					
Septum (mm)	42	10 (6-12)	41	10 (7-14)	0.427
Posterior wall (mm)	41	10 (8-12)	41	9 (7-11)	0.309
LVEDD (mm)	42	50 (41-57)	41	50 (41-55)	0.861
LVESD (mm)	41	32 (18-42)	41	32 (21-41)	0.741
LA parasternal (mm)	42	35 (25-42)	41	35 (25-46)	0.198
LA length (mm)	40	57 (44-69)	41	58 (46-69)	0.764
LA transverse (mm)	24	38 (29-45)	31	39 (29-52)	0.267
Heartbeat (bpm)	34	70 (48-105)	31	66 (48-105)	0.235
Valve dysfunction (n)	42	3 (7.1%)	42	5 (11.9%)	0.457
DIASTOLIC PARAMETERS					
Peak E velocity (cm/s)	42	0.76 (0.58-1.06)	41	0.82 (0.58-1.31)	0.302
Peak A velocity (cm/s)	42	0.59 (0.31-0.99)	41	0.61 (0.35-1.38)	0.097
E/A-ratio	42	1.38 (0.73-2.84)	41	1.35 (0.53-2.30)	0.259
TVI Et (cm/s) ‡	22	12.0 (7-19)	25	10.0 (7-17)	0.002
E/E' ‡	22	6.2 (3.8-12.4)	25	7.6 (4.1-13.1)	0.002
BLOOD PRESSURE ¶					
Systolic (mmHg)	42	129 (107-154)	38	123 (106-153)	0.011
Diastolic (mmHg)	42	74 (58-99)	38	70 (54-106)	0.018

Table 3. Echocardiographic parameters of testicular cancer patients at before and after chemotherapeutic treatment

(*) Wilcoxon signed rank test; (†) Number of patients in which the respective parameters were available; (‡) 19 paired observations available; (¶) 24 hour ambulatory blood pressure recordings

NT pro-BNP

The median level of NT-proBNP increased significantly [N=32; pre-treatment 5 pmol/L (range <5-242); post treatment 18 pmol/L (range <5-114); p=0.034, Figure 2]. One extreme pre-treatment value (242 pmol/L) was in a patient with a TVI Et of 7.0 cm/s pre-treatment.

Levels of NT-proBNP and delta NT-proBNP did not correlate with the echocardiographic parameters pre- and post treatment, and neither correlated with changes in systolic and/or diastolic parameters.

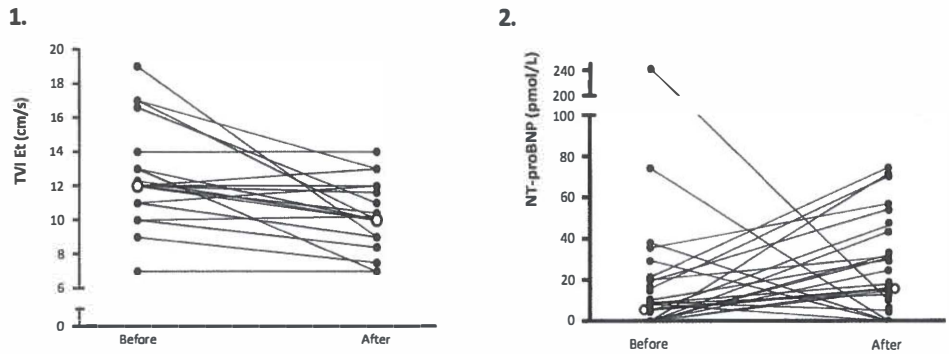

Figure 1. TVI Et in 19 patients pre- and post chemotherapy; open circles represent median values. Median TVI Et pre-treatment 12.0 cm/s (range 7-19), post treatment 10.0 cm/s (range 7-17); p=0.002.

Figure 2. Serum levels of NT-proBNP in 32 patients pre- and post treatment (normal ≤14.75 pmol/L); open circles represent median values. Median NT-proBNP pre-treatment 5 pmol/L (range <5-242); post treatment 18 pmol/L (range <5-114); p=0.034.

Cardiovascular risk factors (CRF)

Before treatment, CRF of the 42 patients consisted of obesity (n=6), smoking (n=14 current smokers; n=5 former smokers) and hypercholesterolemia (n=1). The median BMI of the TC patients was 24.2 kg/m^2 (see Table 2). Nine out of 42 patients (21%) had hypertension on 24-hour ambulatory BP recordings; one of 42 patients was on antihypertensive medication (β-blocker). None had diabetes mellitus.

Seven out of 42 control subjects were obese; the median BMI was 25.0 kg/m2 (Table 2); which was not different from the TC patients (p=0.656). The systolic BP was significantly higher in the patients (p<0.0001); the diastolic BP was not different (p=0.237).

One year post treatment, CRF consisted of obesity (n=8), smoking (current smokers n=11) and hypercholesterolemia (n=3). Four out of 38 patients (11%) had hypertension on 24-hour BP recordings.

Both systolic and diastolic BP decreased post treatment compared to baseline (see Table 3). The pre-treatment systolic blood pressure correlated with delta TVI (R=0.67, p=0.006) and the TVI Et at baseline (R=0.63, p=0.002). E/A-ratio and delta E/A-ratio did not correlate with both systolic and diastolic blood pressure. The E/A-ratio in TC patients pre-treatment correlated with the BMI pre-treatment (R=-0.49, p=0.001), which was also true for pre-treatment TVI Et and BMI (R=-0.50. p=0.019). The WMSI pre-treatment did not correlate with the BMI.

Smokers had a lower E/A-ratio pre-treatment [E/A-ratio smokers 1.31 (range 0.74-2.42) Vs non-smokers 1.56 (0.87-2.41); p=0.039. The TVI Et as well as other echocardiographic parameters, BP and levels of NT-proBNP were not different between smokers and non-smokers.

Events

Nuver et al described acute cardiovascular events in this patient group.[8] During treatment two patients had a myocardial infarction, three had pulmonary embolisms. Omission of patients with cardiovascular events from the analyses did not change the results significantly (data not shown).

DISCUSSION

In this group of testicular cancer patients we observed changes in TVI Et and E/E' within one year after cisplatin-based chemotherapy, representing a deterioration of diastolic cardiac function. Furthermore, serum levels of NT-proBNP increased.

Several authors report changes in cardiovascular status within years to decades after chemotherapeutic treatment for TC,[7,12-14] but little is known of early changes in cardiac function in these patients. Regarding treatment-related cardiotoxicity from various cancer treatments, it was recently postulated that diastolic cardiac function deteriorates prior to the development of systolic dysfunction.[51] In left ventricular dysfunction of various origins, a deterioration of diastolic function can be present in the absence of systolic impairment,[52] and sub-clinical diastolic dysfunction frequently precedes a drop in systolic parameters.[125]

Echocardiography is a frequently used method for assessing cardiac function, which has the advantage that it enables a reliable estimation of diastolic function by means of more recently introduced parameters such as TVI Et and E/E'. Other diastolic parameters, like the E/A-ratio, are largely dependent on preload conditions,[57,126] resulting in significant intra-individual variation.

The TVI Et assesses the velocity of the myocardium at different angles from the mitral valve, instead of blood flow velocities, and is therefore independent of loading conditions,[57,127] resulting in less intra-individual variation. This parameter is considered an important and reliable early predictor for the development cardiac dysfunction in other causes of cardiac disease.[127] From several small studies in adult childhood cancer survivors it appeared to be a valuable parameter in defining diastolic (dys-) function.[59,64,128,129] E/E' is a derivative of TVI Et and the E velocity, thereby including the end-diastolic left ventricular filling pressure in addition to myocardial velocities. This parameter is currently regarded as a valuable non-invasive method for diagnosing diastolic heart failure.[123] Declines in diastolic cardiac function are reflected by increases in E/E' as well as decreases in TVI Et.

The pre-treatment echocardiography parameters in our TC patient group corresponded with the same measurements in an age-matched group of healthy controls. In the patient group a correlation existed between age and pre-treatment E/A-ratio, but not between age and TVI Et or delta TVI Et. This is relevant, as ageing is associated with a physiological decline in diastolic cardiac function.[130] An age-dependent decrease in TVI Et has been demonstrated in healthy controls, but this has only been evaluated in cohorts with larger differences in age and with longer time-intervals.[131]

Several cardiovascular risk factors can lead to declines in diastolic function, including hypertension and obesity.[125] In this study, the pre-treatment systolic BP correlated with delta TVI Et. Contrarily, the BMI did not correlate with the changes in TVI Et. The significant decrease in systolic and diastolic BP on the 24 hour ambulatory recordings was also found in the larger study investigating chemotherapy-induced acute cardiovascular toxicity.[8] The causes for this

finding are not known, but may at least partly be attributed to a relatively high BP before start of treatment, which is confirmed by the finding of a higher systolic BP in the patients compared to the age-matched controls. This high BP might be due to stress before initiation of treatment.

In this study we did not further investigate explanations for this cardiovascular toxicity. The main causes are thought to be related to direct damage to cardiomyocytes and/or the extra-cellular matrix, as well as sub-clinical vascular injury that induces endothelial dysfunction. Furthermore, the presence of pre-treatment elevations in BP may have resulted in impaired relaxation of the left ventricle, thereby leading to diastolic function decline. Of note, the increase in serum NT-proBNP levels is in accordance with the picture of cardiac damage.

It is unknown whether a deterioration of diastolic cardiac function during the first year after chemotherapy for TC will progress to clinically relevant cardiac disease. It can be hypothesised that the hearts of these relatively young patients can compensate for the chemotherapy-induced damage. On the contrary, they are at increased risk of developing an unfavourable cardiovascular risk profile,[13,14,18,19] which can contribute to development of long-term cardiac failure.

In conclusion, we observed significant changes in TVI Et and E/E' within one year after cisplatin-based treatment for TC, indicating a deterioration of diastolic cardiac function. The prognostic significance of this disturbed diastolic function after chemotherapy for future cardiovascular morbidity is not clear, but it might eventually lead to overt cardiac morbidity. Further longitudinal research in TC survivors is needed to obtain more insight in sub-clinical changes in cardiac function.

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Longitudinal changes in cardiac function after cisplatin-based chemotherapy for testicular cancer

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Submitted

Chapter 5

SUMMARY

Background

Cross-sectional studies showed that treatment with cisplatin-based chemotherapy for testicular cancer is associated with an increased incidence of cardiac dysfunction. We investigated longitudinal progression of and contributing factors to cardiac dysfunction in testicular cancer survivors.

Patients and methods

Cardiac assessments were performed before, 10 months (range 7-15) and 6.9 years (range 4.9-9.7) after start of cisplatin-based chemotherapy, consisting of echocardiography [systolic function (Left Ventricular Ejection Fraction, LVEF), diastolic function (myocardial tissue velocities, TVI Et)] and plasma biomarkers [N-terminal Pro-Brain Natriuretic Peptide (NT-proBNP), galectin-3].

Results

In 37 patients [median age 34 years (range 24-51)] the incidence of abnormal TVI Et increased from 0% at baseline, 4.5% at 10 months, to 16.7% at 6.9 years post-chemotherapy (p=0.03). One patient developed LVEF <50%; no other systolic abnormalities occurred. Hypertension, obesity and age were associated with larger decreases in TVI Et. Changes in NT-proBNP and galectin-3 were not related to echocardiographic abnormalities.

Conclusion

In this longitudinal cohort study we observed a gradual decline in diastolic parameters after cisplatin-based chemotherapy for testicular cancer, whereas the rate of systolic dysfunction remains low. The association of larger declines in diastolic parameters with hypertension and obesity stresses the need to monitor and treat cardiovascular risk factors in testicular cancer survivors.

INTRODUCTION

The majority of patients with metastatic testicular cancer can be cured with cisplatin-containing chemotherapy regimens. In the still growing population of testicular cancer survivors the occurrence of long-term treatment-related morbidity, like secondary malignancies and cardiovascular disease (CVD),[7,12-14] is increasingly recognized.[2,6]

Long-term testicular cancer survivors treated with chemotherapy have a higher prevalence of cardiac dysfunction compared to healthy age-matched controls and stage I testicular cancer patients, treated with orchidectomy only.[13,36] In a cross-sectional study in 7-year chemotherapy-treated testicular cancer survivors, diastolic dysfunction was more prevalent than in controls.[36]

Early signs of cardiac damage were reported in testicular cancer patients after cisplatin-based chemotherapy. Within 10 months after start of treatment, cardiac diastolic function measured with tissue Doppler imaging deteriorated compared to baseline, without systolic dysfunction.[132] However, so far it is unknown in what way these early declines in diastolic function progress to cardiac dysfunction over time, as data in this patient population have only been derived from cross-sectional studies.

For anthracycline-related cardiomyopathy subclinical changes in diastolic function are considered to precede the development of systolic failure.[51] In the general population, diastolic abnormalities are associated with an increased rate of cardiovascular morbidity and mortality, including the development of systolic dysfunction.[125,133] In addition to early echocardiographic changes, cardiospecific biomarkers in blood may point to (subclinical) dysfunction prior to clinically overt cardiac failure, and provide reveal mechanisms that contribute to this type of damage. Candidate markers include NT-proBNP,[70] troponin,[71] and galectin-3, a contemporary cardiac fibrosis marker.[134]

Up to now, it is unknown if and in what way early chemotherapy-related declines in diastolic cardiac function progress over time. Therefore, we investigated longitudinal changes in cardiac function in a cohort of testicular cancer patients treated with cisplatin-based chemotherapy. Furthermore, we aimed to estimate which characteristics point to patients at increased risk for cardiac dysfunction in the long term.

PATIENTS AND METHODS

Patients

All patients had been diagnosed with disseminated germ cell cancer. Following orchidectomy, four three-weekly courses of bleomycin (30 USP; days 2, 8, 15), etoposide (100 mg/m^2; days 1-5) and cisplatin (cisplatin 20 mg/m^2; days 1-5) were given, the fourth course without bleomycin. They participated in a study evaluating early cardiovascular changes during cisplatin-based chemotherapy at the University Medical Center Groningen between 2000 and 2004.[8,132] All patients without signs of tumour activity during follow-up were sent a written invitation to undergo assessments at a routine follow-up visit to our outpatient clinic. Patients who needed second line or salvage chemotherapy, or who were diagnosed with other serious co-morbidities were not eligible.

The local ethics committee approved the study, and written informed consent was obtained from all participants.

Measurements

Echocardiography

Transthoracic echocardiography was performed by a sonographer, using the VIVID-7 system (General Electric, Horton, Norway) and a 2.5 MHz probe. Assessments consisted of two-dimensional echocardiography, colour flow mapping and tissue Doppler imaging.[122]

Left ventricular ejection fraction (LVEF, normal >50%) was estimated by two independent experienced technicians, blinded for patient background and each other's estimation. The mean of both outcomes was taken into analysis.

Diastolic function measurements included the following mitral valve inflow velocities: early (E) and late (atrial; A) diastole (E/A-ratio, normal > 1.00), Deceleration Time of the peak early flow (Dt), and Isovolemic Relaxation Time (IVRT). In addition, tissue velocity imaging of early diastole (TVI Et) with colour-coded tissue Doppler imaging was performed. TVI Et was the mean of septal and lateral TVI Et (normal >8.0 cm/s).[123] Colour coded tissue Doppler imaging was done in all patients that were included after January 1st, 2002. Diastolic dysfunction was graded according to recommendations from the American Society of Echocardiography.[123]

Cardiac biomarkers

Blood samples were drawn concomitantly with the cardiac assessments. NT-proBNP (lower limit of detection 5.0 pg/mL) was measured in serum using an immunoassay (Roche Diagnostics, Mannheim, Germany). Galectin-3 was measured in EDTA plasma with an ELISA (BG Medicine, Inc., Waltham, Ma, USA) that measures human galectin-3-levels with high sensitivity (lower limit of detection 1.13 ng/mL), and without crossreactivity with collagens or other members of the galectin-family.[135]

Plasma levels of troponin I were measured during chemotherapy treatment (median 5.5 times), as described earlier.[8] Increases in this cardiomyocyte damage marker were used to explore whether acute increases in troponin I (>0.1 µg/L) were predictive for cardiac abnormalites post-chemotherapy.

Cardiovascular risk factors

Hypercholesterolemia was defined as a fasting cholesterol >6.5 mmol/L and/or use of cholesterol-lowering drugs; diabetes mellitus as a fasting glucose >7.0 mmol/L and/or use of glucose-lowering medication; obesity as Body Mass Index [BMI; calculated by weight(kg)/height(m)2] >27.8 kg/m^2.[136] Blood pressure (BP) was measured in supine position after a minimal rest period of 10 minutes. Hypertension was defined as BP systolic >150 mmHg and/or diastolic >95 mmHg, and/or use of anti-hypertensive medication. For assessment of the metabolic syndrome the American Heart Association/National Heart, Lung and Blood Institute (AHA/NHLBI) classification was used.[137]

Statistical analysis

SPSS software package version 16.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. Non-normally distributed data are represented as median (range), comparisons between groups were tested using the Mann-Whitney U test or Kruskal-Wallis test. For intra-individual changes the Wilcoxon's signed rank test was used. Univariate correlations were calculated with Spearman's correlation (r_s). Univariate linear regression analysis was executed with change in TVI Et during follow-up as dependent variable. Systolic and diastolic blood

pressure, BMI and duration of follow-up were entered as independent variables into the model. Normality of the distribution was checked using normal probability plots of the residuals. In all analyses, two-sided p-values ≤0.05 were considered significant.

RESULTS

Patient characteristics

A total of 65 patients were included in the initial study.[8] In 54 patients baseline echocardiography assessments were available. In 37 of 54 patients cardiac follow-up investigations were performed at median 6.9 years (range 4.9-9.7) after start of chemotherapy (Figure 1). In 29 out of these 37 patients (78.4%) a second echocardiography was performed at a median of 10 months (range 7-15) after start of treatment, as reported previously.[132] Patient- and treatment related characteristics are presented in Table 1. Median age at the follow-up visit was 34 years (range 24-50).

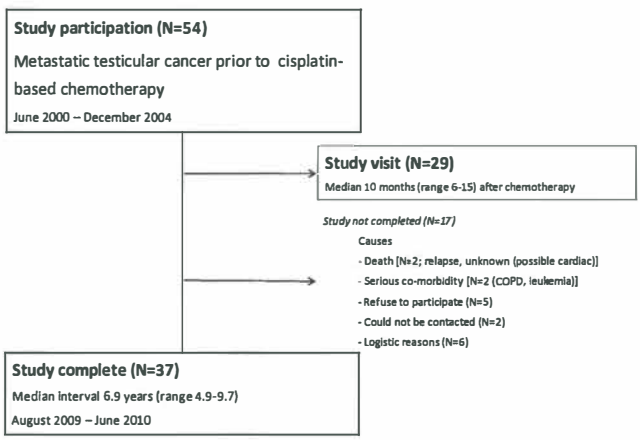

Figure 1. Timing of study investigations and accrual

		Median (range)	N (%)
Number of patients			37
Age at start of treatment (years)		27 (18-44)	
Age at follow-up visit (years)		34 (24-50)	
Interval between assessments			
	Baseline – 1st follow-up visit	10 months (7-15)	
	Baseline – 2nd follow-up visit	6.9 years (4.9-9.7)	
Testicular cancer related characteristics			
	Diagnosis		
	Non-seminoma		37 (100)
	Stage of disease *		
	II		27 (72.8)
	III		2 (5.4)
	IV		8 (21.6)

Table 1 – continued

		N (%)
Testicular cancer related characteristics		
	Prognosis category†	
	Good	25 (67.6)
	intermediate	11 (29.7)
	Poor	1 (2.7)
	Treatment	
	3 BEP	1 (2.7)
	3 BEP + 1 EP	36 (97.3)
	Surgery post-chemotherapy for removal of residual masses	
	RPLND	19 (51.4)
	Thoracotomy	1 (2.7)

Table 1. Patient characteristics. Abbreviations: Bleomycin, Etoposide, Cisplatin (BEP; the number of cycles was based on the prognostic classification), Retriperitoneal Lymph Node Dissection (RPLND).
(*) Royal Marsden classification; (†) According to International Germ Cell Cancer Collaborative Group (IGCCCG)

Cardiovascular status a median of 6.9 years after chemotherapy

Echocardiography findings

At 6.9 years post-chemotherapy the median LVEF was 60% (range 38-65); one patient had an abnormal LVEF. All patients had normal diastolic function according to the American Society of Echocardiography criteria.[123] 17 Six patients (16.7%) had an abnormal TVI Et; six other patients (16.7%) had an abnormal E/A-ratio (Table 2). TVI Et correlated with E/A-ratio (r_s= 0.62, p<0.0001), IVRT (r_s= -0.34, p=0.008) and E/E' (r_s= -0.56, p<0.0001). Besides one patient with previously diagnosed aortic valve regurgitation (Table 3), no other significant valvular disease was observed.

	BASELINE N=37			**10 MONTHS (Range 7-15) N=29**			**6.9 YEARS (Range 4.9-9.7) N=37**		
	Median	Range	Abn(%)	Median	Range	Abn (%)	Median	Range	Abn (%)
TVI Et (cm/s)‡	12.0	9.0-19.0	(0)	10.0*	7.0-17.0	(4.5)	10.5*	6.6-13.6	(16.7*)
E/E'	6.7	3.2-11.1	(0)	7.3*	4.1-13.1	(0)	7.2	4.7-11.6	(0)
E/A ratio	1.48	0.79-2.84	(21.6)	1.35	0.74-2.30	(13.8)	1.37	0.78-2.45	(16.7)
Dt (msec)	181	143-302		198*	118-298		213*	136-282	
IVRT (msec)	76	58-111		88*	64-114		91*†	62-152	

Table 2. Echocardiographic parameters of diastolic function in testicular cancer patients before start of cisplatin-based chemotherapy, median 10 months (range 7-15) and 6.9 years (range 4.9-9.7) after start of treatment. Abbreviations: Tissue Velocity Imaging of the early diastolic (TVI Et), ratio of early atrial blood flow velocity and TVI Et (E/E'), ratio of early and late atrial blood flow velocity (E/A-ratio), Isovolumetric Relaxation Time (IVRT), Deceleration time (Dt), abnormal (abn).
(*) p <0.05 compared to baseline, Wilcoxon Signed Rank test; (†) p <0.05 compared to 10 months post-chemotherapy, Wilcoxon Signed Rank test; (‡) TVI Et was measured from 2002 on; available for longitudinal evaluation in N=26 at baseline; N=22 at 10 months post-chemotherapy.

Cardiac biomarkers

The median serum NT-proBNP level was 13.0 pg/mL (range <5.0-66.0) at 6.9 years post-chemotherapy. At the same time point, the median plasma level of galectin-3 was 11.6 ng/mL (range 6.6-18.7). NT-proBNP and galectin-3 did not correlate with echocardiography parameters (data not shown).

Clinical parameters and cardiovascular morbidity

At follow-up, none of the patients experienced symptoms of cardiac dysfunction, except for a 38-year old patient with dyspnoea on exertion (New York Heart Association class II). On echocardiography hypokinetic wall motion was seen, with LVEF of 38% and TVI Et of 7.7 cm/s. Upon these findings he was referred to a cardiologist. VO_2-max revealed reduced exercise-capacity, at 76% of the predicted value for age and gender. Anti-heart failure medication (β-blockade and ACE-inhibitor) was initiated. In this patient no other explanations for cardiac dysfunction apart from treatment with chemotherapy could be identified, in particular no signs of myocardial infarction.

Four out of 37 patients (10.8%) developed a thrombo-embolic event during chemotherapy.[132] Echocardiography parameters, cardiac biomarkers and cardiovascular risk factors at 10 months and 6.9 years post-chemotherapy were not different in these patients versus patients without acute cardiovascular events. During follow-up, one patient developed a transient ischemic cerebral attack. Three other patients developed non-ischemic cardiovascular morbidity (Table 3). One 35-year old patient died of a possible cardiac arrest one year after chemotherapy, although the cause of death was not confirmed by autopsy.

	Age	Yrs FU‡	CRF	Action	Biomarkers		Echocardiography
					↑ Troponin*	NT-proBNP (pg/mL)†	
Systolic LV dysfunction	27	1	None	Follow-up by cardiologist	+	n.a.	LVEF 55% TVI Et 11.5 cm/s E/A-ratio 2.45 Mild diffuse hypokinesia
Aortic valve insufficiency & stenosis	43	4	Family history	Follow-up by cardiologist	+	66.0	LVEF 60% TVI Et 10.5 cm/s E/A-ratio 1.30 Peak gradient aortic valve 38 mmHg
Paroxysmal atrial fibrillation	27	6	Smoking	Medication resistant; pulmonary vein isolation	-	63.0	LVEF 60% TVI Et 11.4 cm/s E/A-ratio 1.36
Systolic LV dysfunction	38	7 (FU visit)	None	ACE-i and β-blockade	-	25.0	LVEF 38% TVI Et 7.7 cm/s E/A-ratio 1.22 Regional hypokinesia

Table 3 – continued

	Age	Yrs FU‡	CRF	Action	Biomarkers		Echocardiography
					↑ Troponin*	NT-proBNP (pg/mL)†	
Transient ischemic attack	32	5	None	2nd prophylaxis for CVD with statin, ACE-i	+	19.0	LVEF 60% TVI Et 10.6 cm/s E/A-ratio 1.22

Table 3. Characteristics of patients with (cardiovascular) morbidity after cisplatin-based chemotherapy for disseminated testicular cancer. Abbreviations; Cardiovascular Risk Factors (CRF), N-Terminal proBrain Natriuretic Peptide (NT-proBNP), Left Ventricular (LV), Atrial Fibrillation (AF), Cardiovascular Disease (CVD), Angiotensin Converting Enzyme inhibitor (ACE-i), not assessed (n.a.) (*) Rise in troponin I during cisplatin-based chemotherapy; (+) indicates a rise, (-) no change in troponin I levels; (†) Level of NT-proBNP at the follow-up visit median 6.9 years (range 4.9-9.7); (‡) Time-point of diagnosis of cardiovascular morbidity

Cardiovascular risk factors

At 6.9 years post-chemotherapy, six patients (16.2%) had hypertension (4 used antihypertensives, 2 had hypertension detected at the follow-up visit). Five patients had hypercholesterolemia (13.5%). Six patients (16.2%) were obese; the metabolic syndrome was present in seven patients (18.4%). Ten patients (27.0%) were active smokers. Other co-morbidities were hypogonadism in two, diabetes mellitus type 2 in one, and hypothyroidism in one patient.

At 6.9 years post-chemotherapy, TVI Et related to age (rs=-0.65, p<0.0001), diastolic BP (rs=-0.35, p=0.038) and BMI (rs=-0.34, p=0.045). Hypertensive patients had a significantly lower TVI Et [hypertension: median 7.8 cm/s (range 7.2-10.6) vs. no hypertension: median 10.7 cm/s (range 6.6-13.6), p=0.016] and E/A-ratio [hypertension: median 1.06 (range 0.93-1.22) vs. no hypertension: median 1.44 (range 0.78-2.45), p=0.002]. Echocardiography parameters were not different between smokers and non-smokers, or between patients with and without the metabolic syndrome (data not shown). LVEF was not different in patients with or without cardiovascular risk factors (data not shown).

Longitudinal changes in cardiac status

Echocardiography

Compared to baseline, median TVI Et at 10 months and at 6.9 years post-chemotherapy was significantly lower. TVI Et at 6.9 years post-chemotherapy was not different from the visit 10 months post-chemotherapy (Figure 2, Table 2). Compared to baseline, the percentage of patients with a TVI Et <8.0 cm/s increased (Figure 2, Table 2); Dt and IVRT both also significantly increased over time. The median E/A-ratio did not change and median E/E' returned to baseline values.

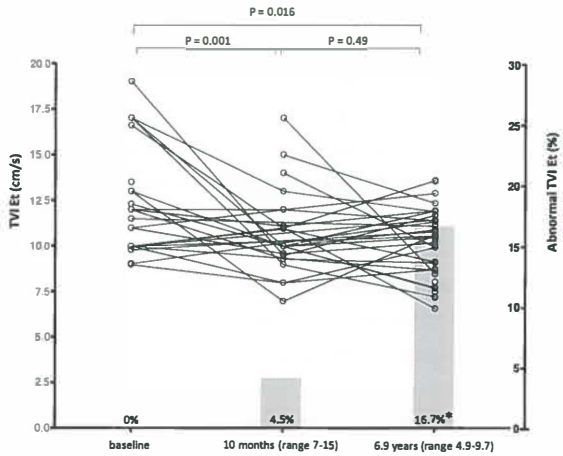

Figure 2. Changes in TVI Et and prevalence of abnormal TVI Et (grey bars) as key parameter for diastolic function, at baseline, median 10 months (range 7-15) and median 6.9 years (range 4.9-9.7) after completion of cisplatin-based chemotherapy for metastatic testicular cancer. P-values are calculated with the Wilcoxon Signed Rank test, asterix indicates significant difference compared to baseline values (p=0.03).

Cardiac biomarkers

The median level of NT-proBNP did not change (Figure 3A). At baseline the median level of NT-proBNP was <5 pg/mL (range <5-114.3), 8 pg/ml (range <5-74.4, p=0.20 compared to baseline) at 10 months post-chemotherapy, and 13 pg/mL (range <5-66, p=0.10 compared to baseline) at 6.9 years post-chemotherapy.

Galectin-3 levels at baseline were not significantly different between IGCCCG prognosis groups [good prognosis: median 12.5 ng/mL (range 7.4-20.1; intermediate prognosis: median 13.4 ng/mL (range 11.6-19.3); poor prognosis: median 15.4 ng/mL (N=1); p=0.79]. At baseline the median level of Galectin-3 was 12.7 ng/mL (range 7.4-20.1), 13.5 ng/ml (range 8.6-22.6, p=0.04 compared to baseline) at 10 months after treatment, and 11.6 ng/mL (range 6.6-18.7, p=0.009 compared to baseline) at 6.9 years post-chemotherapy. Galectin-3 levels were significantly lower at the 6.9 years post-chemotherapy, compared to the two previous assessments (Figure 3B).

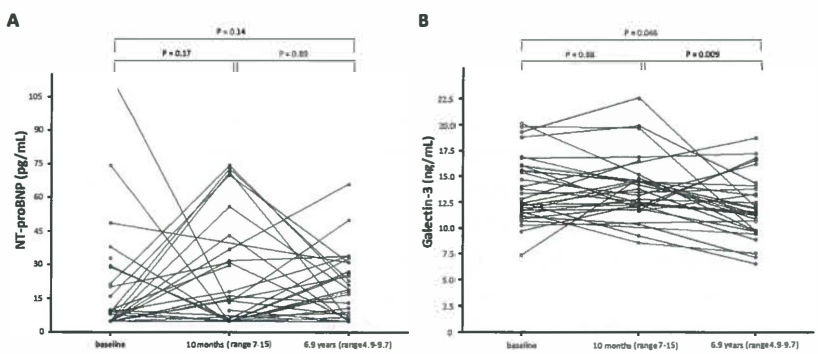

Figure 3. Cardiac biomarkers N-Terminal pro Brain Natriuretic Peptide (NT-proBNP, Figure 3A) and Galectin-3 (Figure 3B), at baseline prior to start of cisplatin-based chemotherapy for metastatic testicular cancer, median 10 months (range 6-15) post-chemotherapy and median 6.9 years (range 4.9-9.7) post-chemotherapy. P-values are calculated with the Wilcoxon Signed Rank test.

Associations between (changes in) echocardiography, cardiac biomarkers and cardiovascular risk factors

Early changes in cardiac biomarkers vs. cardiac status at follow-up

Levels of troponin I were measured during chemotherapy treatment in 31 out of 37 patients (83.8%). Seven of 31 patients (22.5%) had troponin >0.1 µg/L at one or more of sampling points. Three of five patients with cardiovascular morbidity during follow-up had had a rise in troponin I during chemotherapy (Table 3). Post-chemotherapy (changes in) echocardiography parameters, NT-proBNP and Galectin-3 were not different between patients with and without increases in troponin I during chemotherapy (data not shown).

Abnormal systolic or diastolic parameters at follow-up were not associated with levels of, or rise in, NT-proBNP or Galectin-3 (data not shown).

Blood pressure at 6.9 years post-chemotherapy vs. changes in diastolic cardiac status

At 6.9 years post-chemotherapy, systolic BP (r_s=-0.56, p=0.004), diastolic BP (r_s=-0.49, p=0.014) and duration of follow-up (r_s=-0.49, p=0.012) were related to larger declines in TVI Et (ΔTVI Et) from baseline to the follow-up visit. Age was not related to ΔTVI Et (r_s=0.38, p=0.06). Correlations between ΔTVI Et and systolic (r=-0.49, p=0.015) and diastolic BP (r=-0.47, p=0.021) at the visit 6.9 years post-chemotherapy persisted when a partial correlation controlling for follow-up duration was applied.

Regression analysis in all patients showed that systolic BP (b= -0.15 (SE=0.04), p=0.001), diastolic BP (b= -0.21 (SE=0.07), p=0.003), BMI (b= -0.33 (SE=0.15), p=0.041) and follow-up duration (b= -2.24 (SE=0.95), p=0.027) were all independently associated with ΔTVI Et at 6.9 years post-chemotherapy.

Echocardiography at baseline vs. cardiac status at follow-up

No associations existed between echocardiography findings at the successive cardiac evaluations and (changes in) echocardiography parameters at previous assessments (data not shown).

DISCUSSION

This is the first study to perform prospective longitudinal cardiac follow-up in testicular cancer survivors treated with cisplatin-based chemotherapy. Compared to pre-chemotherapy, diastolic cardiac function measured with tissue Doppler imaging deteriorated during 10 months and 6.9 years of follow-up. The observed declines in diastolic cardiac function were most pronounced in patients who developed cardiovascular risk factors like hypertension and obesity. At a median follow-up of 6.9 years after start of chemotherapy, the rate of systolic cardiac dysfunction was low, with 2.7% of the patients having a LVEF <50%.

Different mechanisms may contribute to the observed subclinical declines in cardiac diastolic function. Firstly, chemotherapy may cause direct damage to cardiomyocytes, the extra-cellular matrix and coronary endothelium, resulting in impaired cardiac function. Several studies showed that cisplatin and bleomycin induce endothelial activation and apoptosis.[24-27] Recent preclinical data suggest that cisplatin also damages cardiomyocytes in a direct way through mitochondrial damage and oxidative stress.[138,139] Direct treatment-induced damage to the heart is also suggested by the observed increases in troponin I, a marker for cardiomyocyte

loss,[71] in 23% of patients during chemotherapy. The initial decline in diastolic tissue velocities (TVI Et) and rise in NT-proBNP 10 months post-chemotherapy may also be attributed to direct chemotherapy-induced cardiotoxicity. Secondly, chemotherapy-treated testicular cancer survivors have a higher prevalence of cardiovascular risk factors compared to the general population and stage I testicular cancer patients.[13,14,18,19] Cardiovascular risk factors like hypertension and obesity are associated with secondary cardiac (diastolic) dysfunction, because of increased pre- and afterload demands. In the current study, TVI Et at follow-up and ΔTVI Et were related to high blood pressure, supporting this possible mechanism. The finding that presence of hypertension and obesity is associated with a higher rate of diastolic dysfunction indicates that treatment of these risk factors may be effective to avoid further progression of diastolic dysfunction. In this study we used tissue Doppler imaging to characterize diastolic function. Several diastolic function parameters, e.g. E/A-ratio and IVRT, are largely dependent on preload conditions, resulting in significant intra-individual variation and less reliable interpretation.[57,126] TVI Et, currently regarded as one of the most sensitive diastolic function parameters,[123] is based on myocardial tissue velocities instead of blood flow. Therefore, interpretation of this parameter is less disturbed by hemodynamic conditions, making it a repeatable and solid estimation of cardiac status.

A third mechanism for the decline in cardiac function is ageing, which is associated with a progressive decline in diastolic parameters.[130] During ageing, physiological stiffening of the myocardium leads to delayed relaxation, and a more restrictive left ventricular filling pattern. An age-dependent decrease in TVI Et has been described in healthy controls and hypertensive subjects.[131,140] In this longitudinal follow-up study we did not include a cohort of age matched healthy controls for direct comparison. In healthy controls a decrease in TVI Et of 1 cm/s per decade has been described.[140] Comparing these data to the median ΔTVI Et in our patients, i.e. the median 2.0 cm/s decrease within 10 months post-chemotherapy, indicates that these patients have a larger diastolic function decline than can be expected in the general population.

In the current study, one patient had an abnormal LVEF at 6.9 years post-chemotherapy, whereas one other patient had diffuse hypokinesia directly after completion of treatment. Furthermore, one patient died shortly after completion of treatment, possibly because of a cardiac arrest. In a previous cross-sectional study in 7-year testicular cancer survivors the rate of systolic dysfunction was low too.[36] Collectively, these findings indicate that up to 7 years after cisplatin-based chemotherapy, screening for systolic dysfunction is not warranted.

Galectin-3 is a contemporary marker for cardiac fibrosis that is involved in the progression of heart failure.[134] Patients with disseminated malignancies have higher serum galectin-3-levels than healthy controls, caused by tumour-related cellular proliferation and migration.[141] Although we did not find a relation between galectin-3-levels and pre-chemotherapy metastatic burden, at baseline, galectin-3 may specifically relate to the metastatic malignancy. At 10 months post-chemotherapy, the levels may reflect treatment-induced cardiac fibrosis, as none of the patients had an active malignancy at that time-point. The lower galectin-3 levels at 6.9 years post-chemotherapy implies that cardiomyocyte fibrosis is not likely to contribute to the development diastolic dysfunction in testicular cancer survivors.

Subclinical changes in cardiac biomarkers or echocardiography parameters may precede late clinical dysfunction. When early changes are predictive for later abnormalities, such changes may be used to monitor patients more specifically. In this cohort we did not find associations

between early changes in cardiac biomarkers or echocardiography parameters and the development of cardiac abnormalities during follow-up. Explanations for the lack of predictive power between early, subclinical changes and later cardiac dysfunction may relate to the mild, subclinical cardiac abnormalities at the moment of follow-up. Furthermore, the small sample size results in low power to demonstrate such subtle differences, and the current follow-up duration is still relatively short, as events related to coronary artery disease manifest 15 years or more after chemotherapy. Furthermore, the hypothesis that different phenomena contribute to the development of declines in cardiac function, e.g. direct chemotherapy-induced damage as well as secondary changes related to cardiovascular risk factors, implies that changes in cardiac function do not necessarily develop in a linear manner.

In conclusion, diastolic cardiac function progressively deteriorates in testicular cancer survivors treated with cisplatin-based chemotherapy. Prolonged follow-up in this cohort is needed to estimate the predictive value and clinical implications of this diastolic cardiac dysfunction. The association between (changes in) diastolic function parameters with cardiovascular risk factors stresses the need to monitor and treat these risk factors, in order to minimize the development of late morbidity in successfully treated testicular cancer survivors.

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Raynaud's phenomenon after chemotherapy for testicular cancer is associated with accelerated atherosclerosis

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Submitted

Chapter 6

ABSTRACT

Background

Cross-sectional studies have demonstrated that cisplatin-based chemotherapy for testicular cancer is associated with an increased risk for cardiovascular disease. Longitudinal changes in subclinical parameters for atherosclerosis can reveal mechanisms and biomarkers for late cardiovascular disease.

Methods

Fifty testicular cancer patients underwent vascular evaluations before, median 11 months (8-15) and 6.5 years (3.2-10.1) after bleomycin etoposide cisplatin (BEP) chemotherapy. Cardiovascular morbidity and risk factors, plasma biomarkers for endothelial activation, inflammation and pro-thrombotic state, and carotid Intima Media Thickness (IMT) were determined.

Results

During follow-up, the rate of clinical cardiovascular morbidity was low (2%); 28% developed secondary Raynaud's phenomenon. At 6.5 years post chemotherapy, diastolic blood pressure, endothelial activation markers and carotid IMT increased compared to 11 months. At 6.5 years, median IMT in survivors equalled healthy males, although the age-related IMT progression was higher. Changes in IMT after BEP chemotherapy were associated with increases in serum lipids. Changes in plasma biomarkers were not related to clinical morbidity or changes in IMT. Presence of Raynaud's phenomenon was related to a higher carotid IMT during follow-up, and a higher age-related IMT-increase.

Conclusion

During 6.5-year follow-up after BEP chemotherapy, we observed an increase in subclinical signs of atherosclerosis in testicular cancer survivors. The age-related IMT progression was accelerated when compared to healthy males. The relation between IMT and Raynaud's phenomenon suggests that this phenomenon can serve as a clinical biomarker to identify survivors with augmented atherosclerosis, who may benefit from intervention.

INTRODUCTION

In the growing population of testicular cancer survivors, late treatment-related complications such as cardiovascular disease (CVD)[7,12-14] cause morbidity and impaired quality of life.[2,6] In long-term Dutch testicular cancer survivors an estimated 20-year risk of 18% for CVD was reported, with a 2-fold increased risk for myocardial infarction in non-seminomatous testicular cancer survivors with an attained age below 55 years.[142] In Norwegian long-term testicular cancer survivors, treatment with cisplatin-bleomycin-etoposide (BEP) chemotherapy was associated with a 5.9 times higher risk for myocardial infarction, compared to stage I chemotherapy-naive patients.[16]

Chemotherapy-related endothelial damage contributes to the development of CVD in testicular cancer survivors.[22-27] Subclinical signs of atherosclerosis can be found in these survivors prior to the manifestation of overt CVD.[20,21] Identification of patients with subclinical atherosclerosis at increased risk for cardiovascular complications will be critical to select those who may benefit from interventions that slow or stop the progression towards overt CVD.

Examples of subclinical atherosclerosis markers include structural aspects of vascular status, such as carotid Intima Media Thickness (IMT),[143] and plasma biomarkers for endothelial activation and systemic inflammation. Two cross-sectional studies in 7 year testicular cancer survivors treated with chemotherapy found no difference in median carotid IMT compared to healthy subjects and stage I testicular cancer survivors.[20,21] However, a prospective study showed an increase in carotid IMT of 0.02 mm and elevated levels of von Willebrand Factor (vWF) within 10 weeks after completion of BEP chemotherapy for testicular cancer.[8]

This higher IMT progression rate indicates accelerated atherosclerosis. In the general population, ageing is associated with an increase in carotid IMT over time.[118,144,145] Large increases have been related to an elevated risk for coronary events, and changes in IMT serve as intermediate endpoints for atherosclerosis pro- or regression in intervention studies.[115-117]

This is the first longitudinal follow-up study on subclinical changes in cardiovascular status in testicular cancer survivors treated with BEP-chemotherapy. In this study we aim to evaluate changes in clinical morbidity, cardiovascular risk factors and plasma vascular biomarkers over time. Changes in these parameters are related to changes in carotid IMT, a structural biomarker for atherosclerosis, to identify mechanisms that contribute to atherosclerosis as well as markers that can be used to identify patients at increased risk for CVD.

PATIENTS AND METHODS

Patients

All patients had been diagnosed with disseminated germ cell cancer. They had received four BEP courses lasting 3 weeks each (bleomycin [30 USP, days 2, 8 and 15]; etoposide [100 mg/m^2, days 1-5]; cisplatin [20 mg/m^2, days 1-5], the fourth course without bleomycin). From 2005 on, all patients in the International Germ Cell Cancer Collaborative Group (IGCCCG) good prognosis group received three BEP-courses. They participated in a study evaluating early cardiovascular changes during BEP chemotherapy at the University Medical Center Groningen between 2000 and 2007.[8,132] All patients without signs of tumor activity during follow-up, were sent a written invitation to undergo assessments at a routine follow-up visit to our outpatient clinic. Patients who needed second line or salvage chemotherapy, or who were diagnosed with other serious co-morbidities were not eligible.

The local ethics committee approved the study, and written informed consent was obtained from all participants.
From a previous cross-sectional study in 7-year testicular cancer survivors,[21] data on cardiovascular risk factors and vascular status in a random selection of healthy age-matched males (n = 45) were used for comparison.

Study related investigations
Study related investigations were done at baseline and 1 year after initiation of chemotherapy. All participants had their follow-up visit between June 2009 and August 2010. Study assessments consisted of a standardized medical history, physical examination, fasting blood sampling and measurements of vascular status.

Clinical morbidity
All patients were asked for cardiovascular morbidity, including presence or absence of Raynaud's phenomenon. Development of Raynaud's phenomenon secondary to BEP chemotherapy was defined as occurrence of peripheral discomfort and at least biphasic skin color change in fingers or hands (or toes or feet) after cold exposition, since start of treatment.

Cardiovascular risk factors
Hypercholesterolemia was defined as fasting cholesterol ≥ 6.5 mmol/L and/or use of cholesterol-lowering drugs; diabetes mellitus as a fasting glucose ≥ 7.0 mmol/L and/or use of glucose-lowering medication; obesity as Body Mass Index [BMI; calculated by weight (kg)/height $(m)^2$] ≥ 27.8 kg/m^2. Blood pressure (BP) was estimated as single recording in supine position after a minimal rest period of 10 minutes. Hypertension was defined as BP systolic ≥ 150 mmHg and/or diastolic ≥ 95 mmHg, and/or use of anti-hypertensive medication.
Total cholesterol, High Density Lipoprotein (HDL) and Low Density Lipoprotein (LDL) cholesterol, triglycerides, glucose (all in plasma) and insulin (plasma) were assessed as described elsewhere.[19] HOMA-IR index (Homeostatic Model Assessment for Insulin Resistance = fasting insulin (mU/L) x fasting glucose (mmol/L)/22.5) was calculated as measure for insulin resistance.

Plasma biomarkers for endothelial activation, pro-thrombotic state and inflammation
vWF antigen (reference values 50-150%) was measured in citrate plasma, as described earlier.[21] The soluble adhesion molecules Vascular and Intra-Cellular Adhesion Molecule-1 (sVCAM-1, sICAM-1) were assessed in EDTA-plasma (Fluorkine MAP® Human Adhesion Molecule Base Kit, R&D Systems Europe Ltd., Abingdon, UK). Plasminogen activator inhibitor type 1 (PAI-1) and tissue-type plasminogen activator (tPA) were measured in citrate plasma by ELISA.[18] PAI-1/tPA-ratio was calculated as measure for pro-thrombotic activity. The inflammatory marker high-sensitivity C-reactive protein (hsCRP, reference values < 3.0 mg/L) was assessed in EDTA plasma by nephelometric assay.[21]

Vascular structure: carotid IMT
As parameter for vascular structure, IMT of the right common carotid artery was measured. From three repetitive measurements a mean IMT was calculated, as described previously.[21]

Statistical analysis

Changes in all study parameters were analyzed from the visit one year after initiation of chemotherapy up to the last follow-up visit. The one-year time point was used as a moment of vascular steady state, because the metastatic malignancy and acute chemotherapy-related effects influence hemodynamic and metabolic conditions initially.

SPSS software package version 16.0 (SPSS Inc., Chicago, IL) was used for statistical analyses. Non-normally distributed data were represented as median (range), comparisons between groups were tested using the Mann-Whitney U test or Kruskal-Wallis test. For changes within groups the Wilcoxon's signed rank test was used. Univariate correlations were calculated with Spearman's correlation. Univariate linear regression analysis was executed with IMT at 6.5 years post-chemotherapy as dependent variable. Variables that were significantly correlated with IMT in univariate analyses were entered as independent variables in a multiple linear regression model. Ageing is associated with a significant increase in IMT.[118,144,145] Therefore, to test for an interaction of ageing on the IMT between different groups (i.e. absence or presence of Raynaud's phenomenon, and between patients and controls), a variable for interaction was computed as age*presence/absence of Raynaud's phenomenon, and age*patient/control. Normality of the distribution was checked using normal probability plots of the residuals. P-values ≤ 0.05 were considered to be significant (tested 2-sided).

RESULTS

Patient characteristics

In total 78 patients were included in the initial study, of which 50 patients underwent complete study assessments at median 6.5 years (range 3.2-10.1) after chemotherapy (Fig 1). Study related investigations were performed in 46 out of 50 patients (92%) at a median of 11 months (range 8-15) after initiation of chemotherapy. The median interval between the two follow-up visits was 5.5 years (range 2.2-8.9).

Table 1 summarizes patient and testicular cancer-related characteristics. All patients were initially diagnosed with disseminated testicular cancer. Of all patients, 44 (88%) were treated with four courses of BEP chemotherapy.

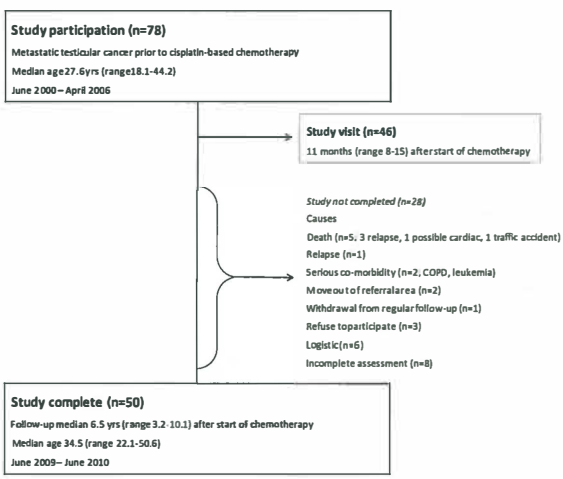

Figure 1. Patient accrual and timing of study-related investigations.

		Median (range)	N (%)
No of patients			50 (100)
Age at start of chemotherapy treatment		28 (18-44)	
Age at follow-up visit		35 (22-51)	
Interval between assessments			
Baseline – 1st follow-up visit months		11 months (8-15)	
Baseline – 2nd follow-up visit		6.5 years (3.2-10.1)	
Testicular cancer related characteristics			
Diagnosis			
	Non-seminoma		50 (100)
*Stage of disease **			
	II		34 (68.0)
	III		3 (6.0)
	IV		13 (26.0)
Prognosis category†			
	Good		33 (66.0)
	intermediate		16 (32.0)
	Poor		1 (2.0)
Treatment			
	3 BEP ‡		6 (12.0)
	4 BEP ‡§		44 (88.0)
Surgery post-chemotherapy			
	Resection of residual masses		24 (48.0)

Table 1. Patient and testicular cancer related characteristics
(*) Royal Marsden classification; (†) According to the International Germ Cell Cancer Collaborative Group (IGCCCG); (‡) BEP; Bleomycin, Etoposide, Cisplatin; (§) In IGCCCG good prognosis: fourth BEP-course without bleomycin

Longitudinal changes in cardiovascular parameters after BEP chemotherapy

Cardiovascular morbidity

During chemotherapy, five out of 50 patients (10%) developed a thrombo-embolic event and one (2%) an arterial event, as reported previously.[21] One patient acutely died of a likely cardiac cause 1 year post-chemotherapy; no autopsy was performed.

During follow-up, one patient (2%) experienced an atherosclerotic vascular event (transient ischemic attack) for which he received medical treatment with a platelet aggregation inhibitor, ACE inhibitor and a statin. Four patients (8%) developed non-ischemic cardiac morbidity. Two had left ventricular dysfunction; in both patients no other explanations for cardiac dysfunction apart from treatment with chemotherapy could be identified, in particular no signs of myocardial infarction. Furthermore, one patient had aortic valve insufficiency and one had paroxysmal atrial fibrillation. All were in regular follow-up by a cardiologist.

Fourteen patients (28%) reported having Raynaud's phenomenon since treatment with BEP-chemotherapy.

Cardiovascular risk factors

At 6.5 years post chemotherapy, median diastolic BP was significantly higher than 11 months post chemotherapy, whereas systolic BP did not change (Table 2). Thirteen patients (26%) had hypertension at 6.5 years post chemotherapy; 10 (20%) newly developed hypertension after chemotherapy. During follow-up the median BMI did not change. Seven patients were obese (14%), of which three (6%) developed obesity after chemotherapy. Median plasma glucose levels increased. One patient (2%) had been diagnosed with type 2 diabetes mellitus after

chemotherapy, for which he received oral glucose-lowering medication. Median levels of total cholesterol did not change. Dyslipidemia was present in six out of 48 patients (12.5%); all newly developed dyslipidemia after chemotherapy. Sixteen patients (32%) were active smokers; all had been smoking prior to start of treatment.

Plasma vascular biomarkers for endothelial activation, pro-thrombotic state and inflammation

Median levels of sICAM-1 and sVCAM-1 were higher at 6.5 years compared to 11 months post chemotherapy (Table 2). The PAI-1/tPA ratio was lower at 6.5 years post chemotherapy, indicating a less pro-thrombotic state. Median plasma levels of vWF and hsCRP did not change.

Vascular structure: IMT

The median IMT was significantly higher at 6.5 years post chemotherapy [0.59 mm (range 0.44-0.94)] than at 11 months post-chemotherapy [median 0.53 mm (range 0.41-0.90), $P < 0.001$] and at baseline [median 0.54 mm (range 0.40-0.96), $P = .004$]. The median annual change in IMT (ΔIMT) from 11 months to 6.5 years post chemotherapy was 0.007 mm/year (range -0.044 – 0.059).

	11 months (range 8-15)		6.5 years (range 3.2-10.1)	
	Median	*Range*	*Median*	*Range*
CARDIOVASCULAR RISKFACTORS				
BMI (kg/m²)	25.3	18.9-38.0	25.5	20.2-41.7
Systolic BP (mmHg)	125	100-170	124	100-180
Diastolic BP (mmHg)	74	60-100	80*	60-105
Glucose	4.7	2.6-5.8	5.5*	3.6-7.4
HOMA-IR	2.4	0.1-9.5	2.2	0.1-10.3
Total cholesterol (mmol/L)	4.7	3.2-7.8	4.8	3.4-7.3
PLASMA VASCULAR BIOMARKERS				
sICAM-1 (ng/mL)	373	159-727	423*	260
sVCAM-1 (ng/mL)	795	312-1165	895*	591
vWF (%)	87	52-237	99	37-204
PAI/tPA ratio	3.2	1.2-14.0	2.6*	0.8-6.7
hsCRP (mg/L)	0.7	0.2-8.9	0.8	0.2-4.7

Table 2. Median levels of cardiovascular risk factors and plasma vascular biomarkers in testicular cancer patients 11 months and 6.5 years after treatment with cisplatin-based chemotherapy. Abbreviations: body mass index (BMI); blood pressure (BP); soluble inter cellular adhesion molecule (sICAM-1); soluble vascular cellular adhesion molecule (sVCAM-1); von Willebrand Factor (vWF); plasminogen activator inhibitor type 1 (PAI-1); tissue-type plasminogen activator (tPA); high-sensitivity C-reactive protein (hsCRP). *(*) P-value <0.05, Wilcoxon signed rank test*

Characteristics associated with IMT at 6.5 years post-chemotherapy and annual ΔIMT

IMT and ΔIMT: associations with clinical parameters

In patients with cardiovascular morbidity during and/or after chemotherapy completion, no differences existed in IMT or in ΔIMT, compared to patients without cardiovascular morbidity (data not shown).

Patients with Raynaud's phenomenon (n = 14) had a higher IMT at 11 months post chemotherapy, whereas IMT at baseline and 6.5 years post chemotherapy was not significantly higher (Fig 2A). Regression analysis in patients with presence or absence Raynaud's

phenomenon showed that age was independently associated with IMT in both groups [Raynaud: b = 0.12 (SE = 0.004), *P* = .009; no Raynaud: b = 0.006 (SE = 0.002), *P* = .002; Fig 2B], with a higher age-related increase in IMT in patients with Raynaud's phenomenon. No differences were found in age, cardiovascular risk factors, (changes in) plasma vascular biomarkers or ΔIMT from 11 months to 6.5 years post chemotherapy between patients with presence or absence of Raynaud's phenomenon (data not shown).

In a multiple linear regression model with IMT as dependent variable, age was independently associated with IMT [b = 0.008 (SE = 0.002), *P* < .0001], whereas presence or absence of Raynaud's phenomenon was not [b = 0.05 (SE = 0.028), *P* = .08]. Introduction of a variable for the interaction between age and presence/absence of Raynaud's phenomenon revealed a median differential age-related IMT-increase of 0.005 mm in patients with Raynaud's phenomenon, which was not significantly different from patients without this symptom [b = 0.005 (SE = 0.004), *P* = .13].

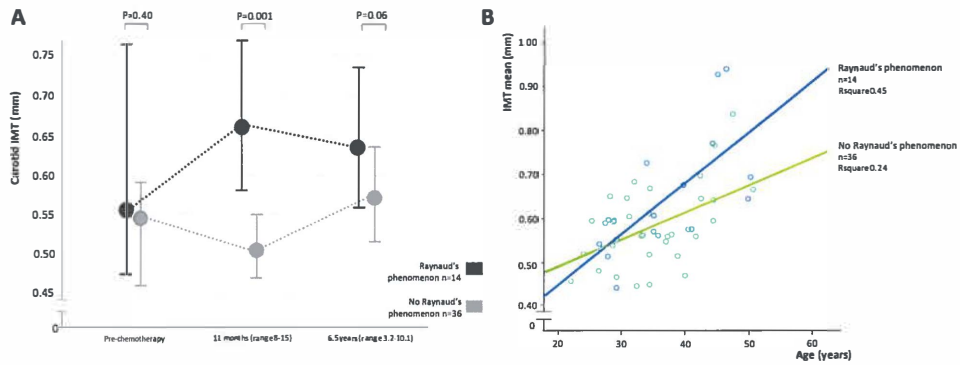

Figure 2A. Fourteen patients (28%) developed Raynaud's phenomenon after treatment with BEP-chemotherapy. Carotid Intima Media Thickness (IMT) is significantly higher at 11 months post-chemotherapy, whereas this difference is not significant at baseline or 6.5 years post-chemotherapy. Dots indicate median values, lines the inter-quartile range; P-values are calculated with Mann-Whitney test.

Figure 2B. Relations between IMT at median 6.5 post-chemotherapy and age in patients with presence or absence of Raynaud's phenomenon secondary to BEP-chemotherapy.

IMT and ΔIMT: association with cardiovascular risk factors

At both post chemotherapy visits, IMT was correlated with age (11 months: r_s = 0.46; 6.5 years: r_s = 0.52; both *P* < .01) and total cholesterol (11 months: r_s = 0.35; 6.5 years: r_s = 0.29; both *P* < .05). At 6.5 years post chemotherapy, IMT was correlated with BMI and lipid levels, but not with BP (Table 3). Patients with a high IMT (highest quartile, i.e. > 0.67 mm) at 6.5 years post-chemotherapy had higher lipid levels at the two post chemotherapy visits (Fig 3). In regression analysis in all patients, age [b = 0.009 (SE = 0.002), *P* < .0001] and total cholesterol [b = 0.031 (SE = 0.015), *P* = .045] were independently associated with IMT at 6.5 years post chemotherapy.

ΔIMT from 11 months to 6.5 years post-chemotherapy was inversely associated with follow-up duration (r_s = -0.32, *P* = .04), but not with age (r_s = -0.04, *P* = .79). ΔIMT was significantly correlated with changes in triglycerides, LDL- and HDL-cholesterol from 11 months to 6.5 years

post-chemotherapy, but not with changes in BMI and BP (Table 4). Presence of cardiovascular risk factors was not associated with differences in ΔIMT (data not shown).

	Testicular cancer survivors		Healthy males	
	R_s with IMT	P-value	R_s with IMT	P-value
Age	0.52	<0.0001	0.47	0.001
BMI (kg/m²)	0.28	0.05	0.35	0.02
Systolic BP (mmHg)	0.21	NS	0.04	NS
Diastolic BP (mmHg)	0.15	NS	0.29	NS
Total cholesterol (mmol/L)	0.29	0.04	0.19	NS
HDL-cholesterol (mmol/L)	-0.17	NS	0.16	NS
LDL-cholesterol (mmol/L)	0.33	0.03	0.11	NS
Triglycerides (mmol/L)	0.33	0.02	-0.05	NS

Table 3. Univariate Spearman correlation coefficients between carotid IMT and determinants for IMT in testicular cancer survivors 6.5 years post-chemotherapy (n=50) and in healthy age-matched males (n=45). Abbreviations: Body Mass Index (BMI); Blood Pressure (BP); High Density Lipoprotein (HDL); Low Density Lipoprotein (LDL); Not Significant (NS; P-value >0.05)

	Median change (range)	R_s with ΔIMT	P-value
Δ BMI (kg/m²)	0.26 (-7.9-6.3)	0.27	0.09
Δ Systolic BP (mmHg)	0.0 (-45-65)	0.05	0.75
Δ Diastolic BP (mmHg)	3 (-25-31)	-0.14	0.40
Δ Total cholesterol (mmol/L)	0.2 (-3.7-1.4)	0.46	0.002
Δ HDL-cholesterol (mmol/L)	0 (-0.8-0.7)	-0.40	0.02
Δ LDL-cholesterol (mmol/L)	0.4 (-2.2-1.1)	0.40	0.018
Δ Triglycerides (mmol/L)	0.1 (-2.4-1.6)	-0.12	0.44

Table 4. Univariate Spearman correlation coefficients between change in carotid Intima Media Thickness (ΔIMT) in testicular cancer survivors from 11 months to 6.5 years post-chemotherapy, and changes in determinants of IMT over the same period. Abbreviations: Body Mass Index (BMI); Blood Pressure (BP); High Density Lipoprotein (HDL); Low Density Lipoprotein (LDL)

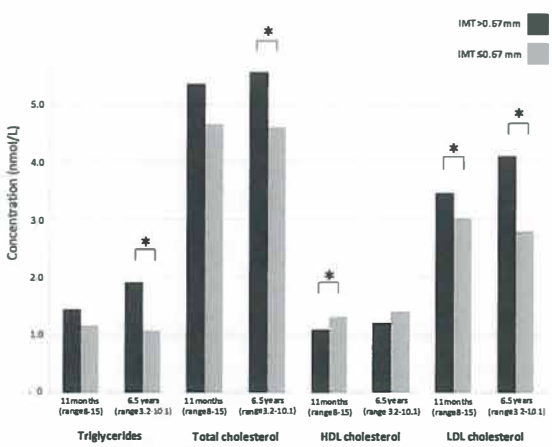

Figure 3. Patients with a high IMT (highest quartile, i.e. >0.67 mm) at 6.5 years post-chemotherapy have a more unfavorable lipid profile at the two post-chemotherapy visits. Bars indicate median concentrations; asterixes indicate significance (P <0.05, Mann-Whitney U test).

IMT and ΔIMT: associations with plasma vascular biomarkers

Absolute levels of, or changes in, none of plasma vascular biomarkers for endothelial activation, pro-thrombotic state and inflammation were associated with ΔIMT or the IMT at 6.5 years post-chemotherapy (data not shown).

Cross-sectional comparison between IMT in 6.5-year testicular cancer survivors vs. healthy males

At 6.5 years post chemotherapy, testicular cancer survivors had a median IMT that was comparable to healthy age matched males [testicular cancer: median 0.59 mm (range 0.44-0.94); controls: median 0.59 mm (range 0.38-0.97); P = .93]. In healthy males, IMT was correlated with age and BMI, but not with lipids or BP (Table 3).

Linear regression analysis showed that age was independently associated with IMT in both testicular cancer survivors [b = 0.09 (SE = 0.02), P < .0001] and healthy males [b = 0.06 (SE = 0.02), P = .003], with a steeper slope for survivors (Fig 4). In a multiple linear regression model with IMT as dependent variable, age [b = 0.007 (SE = 0.001), P < .0001], but not the patient group was significantly associated with IMT [b = 0.015 (SE = 0.019), P = .45]. Next, a variable for the interaction between age and patient/controls was introduced in this model with IMT at 6.5 years post chemotherapy as dependent variable. This revealed a median differential IMT increase of 0.003 mm/year in patients compared to controls, which was not significantly different [b = 0.003 (SE = 0.02), P = .20].

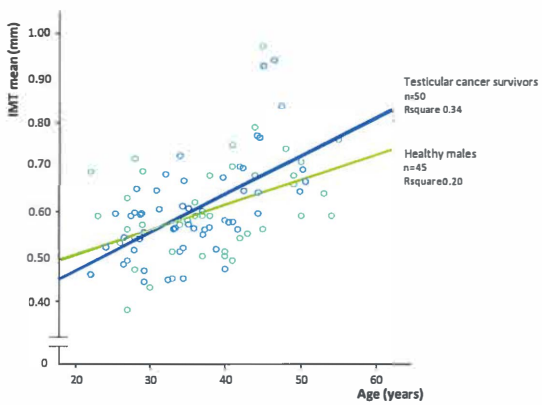

Figure 4. Relations between IMT and age in 6.5-year testicular cancer survivors and in healthy age-matched males.

DISCUSSION

We observed an increase in subclinical signs for atherosclerosis over 6.5 years of follow-up after BEP chemotherapy for testicular cancer. Compared to healthy males, these survivors have signs of accelerated atherosclerosis, with a higher age-related increase in IMT. In patients who developed Raynaud's phenomenon after BEP chemotherapy, carotid IMT and the age-related increase in IMT were higher compared to patients without these complaints. This relation suggests that presence of this symptom can serve as a clinical biomarker that is associated with accelerated atherosclerosis.

So far, data on the incidence of subclinical atherosclerosis in testicular cancer survivors has only been derived from cross-sectional studies. A previous study reported early changes in vascular parameters, i.e. increase in IMT and levels of vWF,[8] suggesting that these patients have accelerated atherosclerosis progression already shortly after BEP chemotherapy.

Carotid IMT is a marker of the systemic atherosclerotic burden that is associated with established cardiovascular risk factors, and an independent predictor for CVD endpoints in the general population.[143] Changes in IMT serve as intermediate endpoint for atherosclerosis pro- or regression in intervention studies[115-117] and large increases in IMT over time are related to an elevated risk for coronary events.[115] Physiological increases in IMT occur in each person during ageing, whereas an enhanced progression rate reflects accelerated atherosclerosis.[27] Overall, the median IMT at 6.5 years post chemotherapy was not different in testicular cancer survivors compared to healthy age-matched males. This finding is in line with data from two cross-sectional studies in 7 year chemotherapy-treated testicular cancer survivors.[20,21] However, we found a higher age-related increase in median IMT in testicular cancer survivors compared to healthy males. This effect was not significant, most likely due to the small sample size. However, this finding is of importance, as a higher age-related increase in IMT was shown to be a sign for accelerated atherosclerosis in patients with familial hypercholesterolemia[146] and systemic lupus erytomatosus.[147]

Because of this accelerated atherosclerosis, interventions that slow this progression may be indicated and should be investigated. A potentially useful target to slow down further progression of atherosclerosis may be treatment with statins, as the annual IMT progression rate in our cohort was related to changes in serum lipids. This rationale is strengthened by the observation that patients with a high IMT at 6.5 years post-chemotherapy had a more unfavorable serum lipid profile already at 11 months post treatment. In a large population based cohort independent associations between ΔIMT and changes in LDL-cholesterol and triglycerides have also been reported,[144] confirming that changes in lipid levels are associated with progression of subclinical atherosclerosis.

Raynaud's phenomenon is characterized by episodic vasospasm of the fingers and/or toes, typically precipitated by exposure to cold.[148] Already in the early 1980's, shortly after introduction of bleomycin- and cisplatin-based regimens for testicular cancer, the occurrence of secondary Raynaud's phenomenon was reported.[10,149] The incidence of 28% in our patients is in line with data from large cohorts, where 20-40% of testicular cancer survivors have Raynaud's phenomenon.[10,150,151] We scored Raynaud's phenomenon with a standardized history form for clinical symptoms, comparable to other studies.[150,151] This way all clinically relevant cases were detected. Additional investigation with cooling plethysmography or nail fold capillary microscopy would be of interest for future studies, to more precisely objectify these complaints and characterize possible associated structural micro vascular abnormalities.

Interestingly we found that patients with Raynaud's phenomenon had a higher carotid IMT 11 months post chemotherapy compared to patients without these complaints, whereas IMT 6.5 years post chemotherapy was not significantly higher. At this last follow-up visit, the median age-related IMT increase in patients with presence of Raynaud's phenomenon was higher, although the effect of this interaction was not statistically different. Nevertheless, these associations suggest that presence of Raynaud's phenomenon in our cohort is associated with objective signs for enhanced atherosclerosis. A study in patients with primary and secondary (due to scleroderma) forms of Raynaud's phenomenon found that carotid artery features were

only different in case of secondary Raynaud's phenomenon.[152] The development of Raynaud's phenomenon secondary to BEP chemotherapy may relate to peripheral endothelial damage. As both IMT as well as Raynaud's phenomenon result from endothelial damage, our findings suggest that presence of Raynaud's phenomenon can be a surrogate biomarker for subclinical atherosclerosis in testicular cancer survivors. The presence of Raynaud's phenomenon may point to patients at increased risk for early development of CVD.

Measurement of plasma vascular biomarkers is a convenient method to monitor for subclinical changes in endothelial status. Moreover, changes in specific markers, e.g. endothelial activation, inflammation or fibrinolysis, may point to mechanisms contributing to chemotherapy-related CVD. Previously, increases in levels of vWF 10 weeks after completion of BEP chemotherapy were reported.[8] In our study endothelial activation markers increased during follow-up, in line with earlier studies reporting on elevated plasma sICAM-1[16] and vWF[15] levels in 7 year testicular cancer survivors. Next to endothelial activation, systemic inflammation is thought to contribute to progression of atherosclerosis. Recently, high levels of hsCRP were associated with a increased risk for later cardiovascular events in longer term testicular cancer survivors.[153] In our study median hsCRP levels did not change over time. Two previous studies reported conflicting findings on levels of hsCRP in chemotherapy treated testicular cancer survivors.[20,21] Therefore this marker seems not suitable as early parameter for subclinical atherosclerosis. Overall, the lack of associations between (changes in) plasma biomarkers and clinical parameters or carotid IMT can be due to relatively short follow-up duration. Furthermore, the heterogeneous mechanisms that contribute to the development of atherosclerosis in testicular cancer survivors, such as direct endothelial damage[22-27] and development of cardiovascular risk factors,[16,18,19] implies that these changes do not necessarily develop in a linear manner.

In conclusion, we observed an increase in subclinical signs for atherosclerosis over 6.5 years of follow-up after BEP chemotherapy for testicular cancer. Testicular cancer survivors have signs of accelerated atherosclerosis when compared to healthy males. In patients who developed Raynaud's phenomenon after BEP chemotherapy, carotid IMT and the age-related increase in IMT were higher compared to patients without this symptom. The relation between IMT and Raynaud's phenomenon suggests that presence of this symptom can serve as a clinical biomarker to identify survivors with augmented atherosclerosis, who may benefit from intervention.

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Metabolic syndrome in testicular cancer survivors develops early after platinum-based chemotherapy

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Submitted

Chapter 7

ABSTRACT

Background: The metabolic syndrome may increase the risk for cardiovascular disease in testicular cancer (TC) survivors.

Objective: To investigate the prevalence of the metabolic syndrome in TC survivors; its development and vascular implications.

Design: Retrospective cohort study.

Setting: A University Medical Center.

Patients: TC survivors treated with platinum-based chemotherapy at the University Medical Center Groningen with a follow-up ≥3 years were eligible (n=370, study I). A subgroup aged <60 years and followed 3-20 years (n=173, study II) was compared to controls from the general Dutch male population (n=1,085).

Measurements: In study I TC survivors were evaluated for development of cardiovascular risk factors. In Study II the subgroup was compared to controls from the general male population for metabolic syndrome prevalence and evaluated for vascular structure and function, and testosterone levels.

Results: In TC survivors (study I) 50% had developed a body mass index >25 kg/m^2, 24% hypercholesterolemia and 30% hypertension, after median follow-up of respectively 1.6, 0.9 and 5.1 years. At median follow-up of 5 years (study II) 25% has the metabolic syndrome (age-adjusted odds ratio (OR) 2.2 (95% CI 1.5-3.3) compared to controls). Survivors with metabolic syndrome have features of inflammation and pro-thrombotic state, increased carotid artery intima-media thickness and decreased baroreflex sensitivity. Survivors with testosterone levels <15 nmol/L (22%) have an increased risk for the metabolic syndrome (OR 4.1, 95% CI 1.8-9.3).

Limitation: Retrospective design of this study.

Conclusion: The study suggest that the metabolic syndrome occurs at earlier age in TC survivors treated with chemotherapy compared to controls and is accompanied by inflammatory and pro-thrombotic features, and signs of atherosclerosis. This emphasises the need for guidelines on early screening and treatment.

INTRODUCTION

Cardiovascular disease is a frequent late effect after treatment for testicular cancer with an estimated 20-year risk of about 18% in Dutch testicular cancer survivors.[103] Coronary heart disease is particularly common (estimated 20-year risk 10%) with approximately two-fold increased risk for myocardial infarctions in non-seminomatous testicular cancer survivors with attained age <55 years.[103] Consequently, cardiovascular disease represents a threat to life expectancy and quality of life of a large proportion of survivors. Both mediastinal radiotherapy[103] and platinum-based chemotherapy regimens[12,103] are associated with increased cardiovascular disease risk compared to treatment with surgery alone. This increased cardiovascular disease risk may be related to accelerated atherosclerosis.[21]

Platinum-based chemotherapy is associated with increased prevalence of excessive weight gain,[154] dyslipidemia[13], hypertension[13,154] and insulin resistance.[13] These factors are the core components of the metabolic syndrome, which is associated with increased risk for atherosclerotic disease in the general population.[155] Central obesity with visceral adipose tissue deposition may have an important role. Its hyperlipolytic state and excess of free fatty acids contribute to insulin resistance. In addition, visceral adipose tissue produces pro-inflammatory and pro-thrombotic factors.[155,156]

We previously reported on increased prevalence of the metabolic syndrome in testicular cancer survivors compared to healthy controls at median 7 years after chemotherapy.[19] In a Norwegian cohort of testicular cancer survivors with median follow-up of 11 years only survivors treated with cumulative cisplatin dose >850 mg showed increased risk for metabolic syndrome compared to healthy controls.[157] In both studies testosterone levels were inversely associated with the metabolic syndrome.[18]

Information on the metabolic syndrome and its effects on vascular function and development of overt cardiovascular disease in cancer survivors is scarce. Insight into etiological and/or contributing factors may help to identify survivors at increased risk for the metabolic syndrome.[31] In order to develop guidelines for follow-up on cardiovascular risk factors in testicular cancer survivors, information is also needed on development over time of such factors.[6]

In this study we present data on cardiovascular risk factors and the metabolic syndrome in long-term (≥3 years) non-seminomatous testicular cancer survivors treated with platinum-based chemotherapy: the development over time of cardiovascular risk factors (study I), the prevalence of metabolic syndrome compared to the general male population, its associated features, and implications for vascular structure and function, and its relation to gonadal function (study II).

METHODS

Patients

Patients were selected from our cohort of long-term survivors of disseminated non-seminomatous testicular cancer treated with platinum-based chemotherapy at University Medical Center Groningen, between January 1977 and April 2004 (n=439) (Figure 1). For the retrospective evaluation of the development of cardiovascular risk factors (study I) patients had to have a follow-up duration of ≥3 years. Patients with coronary heart disease [defined as myocardial infarction or coronary artery disease (proven by angiography, or by treatment with

angioplasty or bypass surgery)] before start of chemotherapy were excluded. The metabolic syndrome and its associated features (study II) were cross-sectionaly assessed in a subgroup of study I. Inclusion criteria were attained age <60 years and follow-up of maximum 20 years. Exclusion criteria were history of cardiac disease before or after chemotherapy, radiotherapy to head, neck and/or mediastinum, and participation in a previous study on cardiovascular risk profile.[13] The medical ethical committee approved the study and participants gave written informed consent.

Control population

Control data concerning the metabolic syndrome in the general population were available from two studies: men 28-60 years old from a representative sample of the Groningen population of the Prevention of Renal and Vascular End-stage Disease (PREVEND) study[158,159] and, in order to cover also for young testicular cancer survivors, men 18-42 years old participating as sibling controls to a study on late cardiovascular effects in childhood cancer survivors. Controls with a history of myocardial infarction or coronary artery disease were excluded. In 1,020 out of 1,120 participants of the PREVEND study and in 65 out of 68 in the childhood cancer survivor study, sufficient cross-sectional data were available on waist circumference, blood pressure, fasting lipid and glucose levels and medication use, to allow for evaluation of presence or absence of the metabolic syndrome. The resulting control group existed of 1,085 men with a median age of 44 years (range 18-59).

Evaluation of the development of cardiovascular risk factors (Study I)

Medical records, updated from general practitioners' files, were evaluated for disease- and treatment-related characteristics and follow-up data. Cardiovascular risk factors overweight, hypercholesterolemia and hypertension were retrospectively evaluated for presence pre-chemotherapy and development during follow-up. With respect to overweight, body mass index (BMI; calculated by weight(kg)/height(m)2) >27.8 kg/m^2 was recorded, the latter corresponding with an earlier US National Institutes of Health definition for overweight[136] and used in the early phase of establishment of the cohort.[13] Hypertension was defined as blood pressure systolic >150 mmHg and/or diastolic >95 mmHg[13,160] and/or use of antihypertensive drugs, and hypercholesterolemia as non-fasting cholesterol levels >6.5 mmol/L at ≥3 separate time points and/or use of cholesterol-lowering drugs. Follow-up duration was calculated from start of chemotherapy to last assessment of each risk factor and in case of overweight, hypertension or hypercholesterolemia also to date of this diagnosis.

Assessment of the metabolic syndrome and its associated features (Study II)

Cross-sectional evaluation consisted of a medical history focused on medication use, smoking behavior and family history for cardiovascular disease (positive in case of parent or brother/sister with coronary heart disease, cardiac death or cerebrovascular accident at age <60 years) and assessment of weight, height, waist circumference (at umbilical level), hip circumference (at broadest part) and blood pressure.

Fasting blood samples were analyzed for HDL-cholesterol and triglycerides (plasma), glucose (plasma) and insulin (serum). Leptin and adiponectin were determined by ELISA (#EZHL-80SK and #EZHADP-61K, Linco Research, St. Charles, MO, US, EDTA plasma). In addition, markers for inflammation, pro-thrombotic state and endothelial activation were determined. High-

sensitivity C-reactive protein (hsCRP, serum), fibrinogen, Von Willebrand Factor (vWF) antigen, Plasminogen Activator Type-1 (PAI-1) antigen, and tissue-type Plasminogen Activator (t-PA) antigen (citrate plasma) were measured as described previously.[21] Urinary albumin excretion and creatinine clearance were determined from a 24-h urine sample.[21]

For assessment of the metabolic syndrome the American Heart Association/National Heart, Lung and Blood Institute (AHA/NHLBI) classification[137] was used with presence of the metabolic syndrome in case of ≥3 of the following criteria: central obesity (waist circumference ≥102 cm), high triglycerides (≥1.7 mmol/L or medication), low HDL-cholesterol (<1.03 mmol/L or medication), high blood pressure (systolic ≥130 mmHg or diastolic ≥85 mmHg or medication), and high glucose (≥5.6 mmol/L or medication). In addition, waist/hip ratio was calculated as measure for central obesity, HOMA-IR index (Homeostatic Model Assessment for Insulin Resistance = fasting insulin (mU/L) x fasting glucose (mmol/L)/22.5) for insulin resistance and PAI-1/tPA ratio for pro-thrombotic state. As potential risk factor gonadal function was analyzed by measuring serum total testosterone, luteinizing hormone (LH) and follicle-stimulating hormone (FSH).

As measure for cardiovascular autonomic function, baroreflex sensitivity (BRS) at rest in supine position was determined.[36] To assess vascular structure and function, the intima-media thickness (IMT) of the common carotid artery was measured and carotid distensibility, compliance and stiffness calculated, as described previously.[21]

Statistical methods

The cumulative risk for cardiovascular risk factors in testicular cancer survivors (study I) was plotted according to the Kaplan-Meier procedure. Presence of a risk factor at last assessment was considered an event, with follow-up duration calculated from start of chemotherapy to diagnosis of the risk factor. When the risk factor was already present pre-chemotherapy, follow-up duration was set to zero. Median follow-up until development of each risk factor was calculated for testicular cancer survivors in whom the risk factor developed after completion of chemotherapy.

The risk for the metabolic syndrome and its components in testicular cancer survivors (study II) was compared to general population data by binary logistic regression analysis with adjustment for age. Testicular cancer survivors with metabolic syndrome were compared to survivors without metabolic syndrome with respect to associated features, vascular structure and function, and gonadal function using the non-parametric Mann-Whitney U test for continuous data and the X_2 test for categorical data. The association between metabolic syndrome and markers for inflammation, pro-thrombotic state, endothelial activation, and vascular structure and function was further analyzed using linear regression analysis with metabolic syndrome as independent variable and adjustment for age. The effect of gonadal function on the risk for the metabolic syndrome and its components was tested with binary logistic regression analysis. Receiver-Operator-Characteristic curve analysis was used to estimate the optimal cut-off value for total testosterone levels in order to differentiate between persons with and without metabolic syndrome. Statistical analyses were performed with SPSS for Windows 16.0 (SPSS Inc., Chicago, IL, US). For all analyses, a 2-tailed p-value <0.05 was considered significant.

RESULTS

Study population characteristics

The original cohort of 439 non-seminomatous testicular cancer patients treated with platinum-based chemotherapy includes 370 patients with absence of pre-treatment history of coronary heart disease and follow-up ≥3 years (study I) (Figure 1). From the subgroup of 212 patients approached for participation in the study on the metabolic syndrome (study II), 173 patients (82%) gave informed consent. Baseline disease- and treatment-related characteristics and follow-up data for the two study populations are shown in Supplementary table 1.

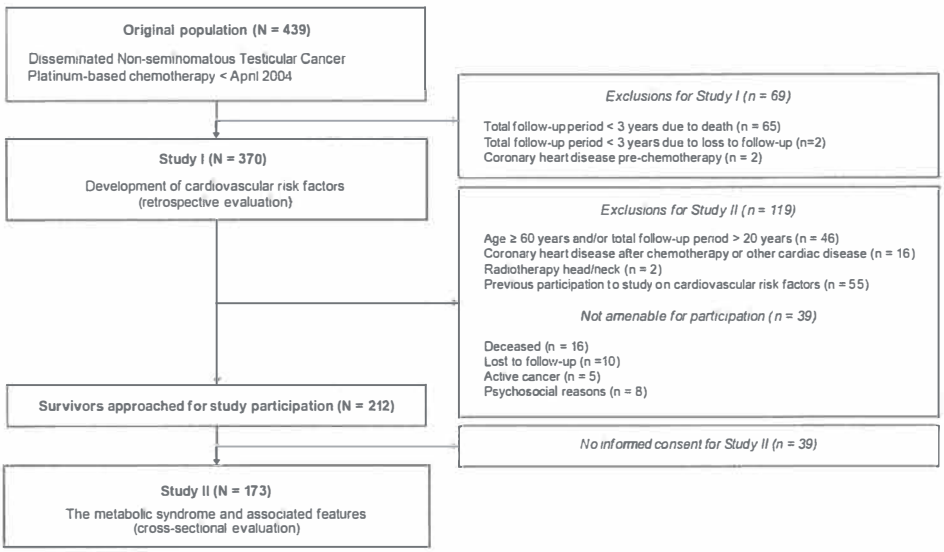

Figure 1. Flow diagram of included patients.

Development of cardiovascular risk factors (Study I)

Assessments of overweight, hypercholesterolemia and hypertension were available until median follow-up of 9.1 years (range 0.2-28.9). At last assessment the prevalence of risk factors was: BMI >27.8 kg/m^2 85/359 (24%, new in 15% of the patients compared to pre-chemotherapy), hypercholesterolemia 87/361 (24%, new in 14%) and hypertension 106/359 (30%, new in 23%).

Figure 2 shows cumulative risks for the risk factors from start of chemotherapy. Median time to the development of BMI >27.8 kg/m2 is 1.7 years (range 0.2-28.4), for hypercholesterolemia 0.9 years (range 0.2-22.4) and for hypertension 5.1 years (range 0.2-21.2).

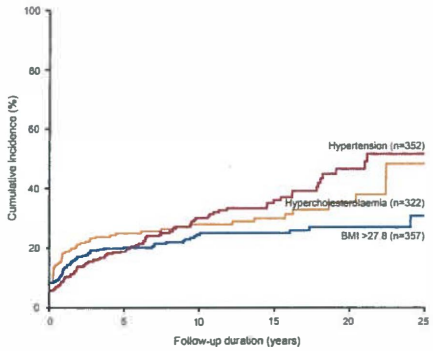

Figure 2. Cumulative risk of cardiovascular risk factors overweight, hypercholesterolemia and hypertension from start of chemotherapy (study I). Data are shown for patients in whom data on the concerned risk factors at start of chemotherapy are available.

The metabolic syndrome and associated features (Study II)

Prevalence

At median follow-up of 5 years (range 3-20) and attained age of 37 years (range 19-59) the prevalence of the metabolic syndrome was 44/173 (25%). The prevalence increases with ascending age, from 13.3% in survivors 18-30 years old to around 35% in survivors 40-60 years old (Figure 3A). High blood pressure is the most frequent component of the metabolic syndrome (59%), followed by low HDL-cholesterol (44%), high triglycerides (29%), central obesity (17%) and high glucose levels (14%) (Figure 3B; Table 1). All components tend to increase in prevalence with age.

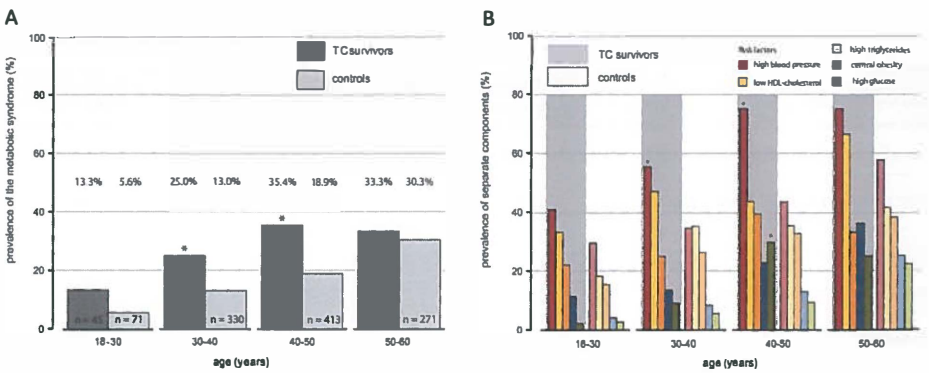

Figure 3. Prevalence of the metabolic syndrome in testicular cancer survivors and in controls according to age category (A) and prevalence of its separate components (B) (study II).
(*) p <0.05 compared to controls (X_2 test)

Adjusted for age, testicular cancer survivors show an increased risk for the metabolic syndrome compared to the general population with an odds ratio of 2.2 (95% confidence interval 1.5-3.3) (Table 1). This effect corresponds with 12.9 years' increase in age compared to the control population. Testicular cancer survivors especially have an increased risk for high blood pressure (Table 1).

Dependent variable	Prevalence	Odds ratio	95% CI	p-value
Metabolic syndrome	44/173 (25%)	2.2	1.5-3.3	0.0001
Separate components				
High blood pressure	100/171 (59%)	2.5	1.8-3.6	<0.0001
Low HDL-cholesterol	76/173 (44%)	1.7	1.2-2.3	0.003
High triglycerides	50/173 (29%)	1.2	0.8-1.7	0.424
Central obesity	29/170 (17%)	1.9	1.2-3.1	0.005
High glucose	24/170 (14%)	2.3	1.4-3.7	0.002

Table 1. Prevalence of and risk for the metabolic syndrome and its separate components after chemotherapy for testicular cancer (study II, N=173). The risks are expressed as age-adjusted odds ratios compared to the control population with 95% confidence interval (95% CI) and p-value (binary logistic regression).

Metabolic syndrome associated features

There is no difference in smoking behavior and family history for cardiovascular disease between survivors with and without metabolic syndrome. Survivors with metabolic syndrome have more central obesity, reflected by a higher waist/hip ratio, higher plasma leptin and lower plasma adiponectin, and increased HOMA-IR index (Table 2).

The association of metabolic syndrome with inflammation, pro-thrombotic state, endothelial activation, and signs of vascular damage was analyzed with adjustment for age. In presence of metabolic syndrome pro-thrombotic markers fibrinogen and PAI-1/tPA ratio are increased. Levels of hsCRP and vWF do not differ (Table 3).

Characteristics *	Metabolic syndrome present (44)	Metabolic syndrome absent (129)	p-value†
Survivor characteristics and cardiovascular risk factors			
Age at start chemotherapy (years)	29 (18–48)	26 (16–52)	0.086
Follow-up duration (years)	7 (3–20)	5 (3–20)	0.046
Age at study participation (years)	40 (23–57)	35 (19–59)	0.005
Smoking habits			
Current smoker	14/44 (32%)	51/128 (40%)	0.546
Ex-smoker	11/44 (25%)	24/128 (19%)	
Life-long nonsmoker	19/44 (43%)	53/128 (41%)	
Positive family history CVD	6/42 (14%)	17/123 (14%)	1.000
Metabolic features			
BMI (kg/m^2)	28.4 (22.9–38.7)	24.0 (17.4–38.8)	<0.0001
Waist/hip ratio	0.99 (0.83–1.12)	0.92 (0.80–1.15)	<0.0001
Leptin (ng/mL)	12.77(2.37–43.29)	3.71 (0.24–66.12)	<0.0001

Table 2 – continued

Characteristics *	Metabolic syndrome present (44)	Metabolic syndrome absent (129)	p-value†
Metabolic features			
Adiponectin (µg/mL)	5.00 (2.04–11.19)	7.23 (2.76–17.40)	<0.0001
HOMA-IR index	3.30 (1.56–23.86)	2.19 (0.20–2.04)	<0.0001
Gonadal function			
Testosterone supplementation	1/44 (2%)	8/129 (6%)	0.451
Total testosterone (nmol/L)‡	15 (9–31)	18 (4–37)	<0.0001
Testosterone <15 nmol/L‡	19/43 (44%)	17/121 (14%)	0.0002
FSH (U/L)‡	16.80 (4.34–47.50)	13.90 (2.38–7.50)	0.452
LH (U/L)‡	6.42 (2.47–18.70)	5.45 (1.59–33.10)	0.190

Table 2. Comparison between testicular cancer survivors with the metabolic syndrome and testicular cancer survivors without the metabolic syndrome with respect to survivor characteristics, cardiovascular risk factors, metabolic features and gonadal function (study II).
(*) Median (range) or n/N (%); (†) Mann-Whitney U test for continuous data and X2 test for categorical data; (‡) For testicular cancer survivors not receiving testosterone supplementation

Testicular cancer survivors with metabolic syndrome do not differ with respect to creatinine clearance or urinary albumin excretion. In contrast, presence of metabolic syndrome is associated with lower baroreflex sensitivity at rest and increased carotid artery intima-media thickness (Table 3).

Gonadal function

Median total testosterone level is lower in presence of metabolic syndrome (excluding survivors with testosterone supplementation (n=9)) (Table 2; Supplementary figure 1). A cut-off value of total testosterone <15 nmol/L was considered best discriminating for the metabolic syndrome (area under the Receiver-Operator-Characteristic curve 0.65 (95% CI 0.55-0.74), area under the Receiver-Operator-Characteristic curve of age 0.65 (95% CI 0.55-0.75). Unsupplemented total testosterone <15 nmol/L is associated with an age-adjusted odds ratio of 4.1 (95% CI 1.8-9.3; p=0.001) for the metabolic syndrome (Supplementary Table 2). Low testosterone shows association with central obesity (odds ratio=5.7 (95% CI 2.3-14.1); p<0.001) and high glucose levels (odds ratio=2.9 (95% CI 1.1-7.5); p=0.031).

Associated features*		Metabolic syndrome present (44)	Metabolic syndrome absent (129)	B (age-adjusted)†	p-value†
Markers for inflammation, pro-thrombotic state and endothelial cell activation					
hsCRP (mg/L)		1.6 (0.2 – 13.4)	1.1 (0.26 – 31.8)	0.36 (-0.75 – 1·47)	0.52
vWF (%)		98 (28 – 220)	96 (37 – 296)	-5 (-21 – 10)	0.52
Fibrinogen (g/L)		3.2 (1.5 – 5.0)	2.8 (1.2 – 6.3)	0.24 (0.01 – 0.46)	0.04
tPA (ng/mL)		11.0 (3.7 – 21.0)	6.5 (1.5 – 21.0)	3.5 (2.3 – 4.6)	<0.0001
PAI-1 (ng/mL)		57 (8 – 312)	19 (3 – 62)	42.1 (29.0 – 55.3)	<0.0001
PAI-1/tPA ratio		5.4 (1.5 – 31.6)	3.2 (0.5 –17.1)	3.1 (1.7 – 4.6)	<0.0001
Vascular function and structure					
Creatinine clearance (mL/min)	n=169	122 (36 – 240)	119 (44 – 245)	9 (-2 – 21)	0.12
Albumin excretion (mg/24h)	n=169	5.5 (0.1 – 615.3)	4.6 (0.1 – 345.4)	7.67 (-12.67 – 28.09)	0.46
BRS (ln) at rest (ln(ms/mmHg))	n=166	2.3 (0.8 – 3.6)	2.6 (0.8 – 3.6)	-0.21 (-0.41 – -0.01)	0.04
Carotid IMT (mm)	n=157	0.62 (0.45 – 1.05)	0.57 (0.38 – 1.12)	0.044 (0.003 – 0.084)	0.03
Carotid compliance (µm/mmHg)	n=142	8.36 (4.29 – 17.85)	10.74 (3.70 – 30.67)	-1.45 (-3.02 – 0.13)	0.07
Carotid distensibility (%)	n=144	6.62 (3.36 – 9.78)	7.74 (3.41 – 16.84)	-0.62 (-1.37 – 0.14)	0.11
Carotid stiffness	n=137	7.22 (3.92 – 13·24)	6.50 (3.24 – 12.51)	0.33 (-0.42 – 1.08)	0.39

Table 3. Markers of inflammation, pro-thrombotic state, endothelial cell activation, and vascular function and structure (study II) according to presence or absence of the metabolic syndrome.
(*) Median (range); (†) Co-effficient B (95% confidence interval) and p-value for the association between the metabolic syndrome and concerning feature (linear regression with metabolic syndrome as independent variable and adjustment for age)

DISCUSSION

Our cross-sectional assessment of the metabolic syndrome (study II) shows that this syndrome and its components central obesity, low HDL-cholesterol, high blood pressure and high glucose levels are more prevalent in testicular cancer survivors than in the general population. At median follow-up of 5 years 25% of the survivors have the metabolic syndrome. The risk for

the metabolic syndrome in testicular cancer survivors is 2.2 (95% CI 1.5-3.3) times higher than in the general population, corresponding with the risk of men 12.9 years older. This suggests development of metabolic risk factors at earlier age.

Retrospective evaluation of the development of cardiovascular risk factors (study I) shows that overweight and hypercholesterolemia develop mainly within the first 5 years after platinum-based chemotherapy, after median follow-up of 1.7 years for BMI >27.8 kg/m^2 and 0.9 years for hypercholesterolemia. The cumulative risks of overweight and hypercholesterolemia show a plateau after a follow-up period around 10 years. In contrast, hypertension develops later, after median follow-up of 5.1 years, and does not show a plateau with prolonged follow-up. The later appearance of hypertension may be related to its development secondary to other metabolic risk factors. Moreover, hypertension may reflect the long-term effect of direct chemotherapy-induced endothelial damage.

A considerable proportion of the testicular cancer survivors with overweight, hypercholesterolemia or hypertension at the end of follow-up already had these risk factors at start of chemotherapy. Unfortunately, it is unknown whether the pre-chemotherapy prevalence of these factors is representative for the age-matched general population. On the one hand, presence of cancer may contribute to lower cholesterol levels and lower BMI as previously described.[161] On the other hand, the metabolic syndrome and its components are increasingly recognized as risk factors for the development of several cancer types[162] and may be consequently overrepresented in cancer patients.

Although we have no longitudinal data on the development over time of cardiovascular risk factors in the general population, our data suggest that the metabolic syndrome develops at earlier age in testicular cancer survivors (study II) and that development of cardiovascular risk factors can already be observed during the first years of follow-up (study I).

In line with previous studies we have found an association between total testosterone levels and metabolic syndrome in testicular cancer survivors.[18,19] In the general population low testosterone levels are predictive for the development of metabolic syndrome.[163-165] Our data show that survivors with unsupplemented total testosterone <15 nmol/L (22% of the evaluated survivors) have an approximately 4 times increased risk for the metabolic syndrome, particularly associated with central obesity. We hypothesize that decrease in testosterone levels and secondary central, visceral fat deposition have an important role in the development of the metabolic syndrome. Unsupplemented testosterone <15 nmol/L also shows an association with high glucose levels. This corresponds with the independent association of low to normal testosterone levels with insulin resistance, as observed in older men.[166]

Testicular cancer survivors with metabolic syndrome show features of central, visceral fat deposition: increased waist-hip ratio, elevated levels of leptin (adipose tissue hormone giving feedback at hypothalamic level on energy intake and expenditure), decreased levels of adiponectin (adipose tissue hormone with anti-inflammatory and insulin-sensitizing effects), and insulin resistance (increased HOMA-IR index). Decreased adiponectin levels and increased fibrinogen levels are associated with an inflammatory state. The observed increase in both fibrinogen and PAI-1/tPA-ratio may contribute to a pro-thrombotic state. Low-grade inflammation and pro-thrombotic state are closely linked in the patho-biology of atherosclerosis.[167,168] The observed association with increased carotid intima-media thickness and decreased baroreflex sensitivity underscores the potential clinical relevance of the

metabolic syndrome in testicular cancer survivors. Increase in intima-media thickness is an accepted marker of early atherosclerosis[169] and decrease in baroreflex sensitivity[170,171] has also been associated with atherosclerosis. Both are considered unfavourable predictors for cardiovascular disease.

Although our data suggest a role of the increased prevalence of cardiovascular risk factors in the development of cardiovascular disease in testicular cancer survivors treated with platinum-based chemotherapy, prolonged follow-up is needed to assess whether subclinical signs of atherosclerosis eventually progress to overt cardiovascular disease. The homogeneity of the study population, most survivors having been treated with the current standard regimen BEP, excludes analysis of direct toxic effects of chemotherapy on the vasculature. Cisplatin as well as bleomycin can induce endothelial damage.[24,172,173] Since cisplatin in plasma remains detectable up to 20 years after chemotherapy[28] and may still be partially in reactive form,[174] we hypothesise there is an ongoing process of platinum-induced direct vascular toxicity.

The current observations on cardiovascular risk factors and metabolic syndrome in long-term testicular cancer survivors suggest that detection and timely treatment from start of chemotherapy and during follow-up may contribute to reduction of cardiovascular disease risk. Overweight and hypercholesterolemia can be detected mainly within the first 5 years after chemotherapy. However, the risk for hypertension, that may also be a sign of developed vascular damage, extends beyond a follow-up period of 10 years. Therefore, extended follow-up, in close collaboration with primary care physicians, seems necessary.

By implementing prolonged follow-up it will become clear whether testicular cancer survivors with metabolic syndrome will have a higher cardiovascular disease risk than testicular cancer survivors without, and than individuals with metabolic syndrome in the general population. If previous treatment for testicular cancer turns out to be an additional cardiovascular risk factor, treatment indications for the metabolic syndrome may be more stringent for testicular cancer survivors. In addition, a randomised, placebo-controlled trial may help to evaluate whether testosterone supplementation contributes to prevention or treatment of metabolic syndrome in testicular cancer survivors.

In conclusion, the metabolic syndrome is more prevalent and appears to develop at earlier age in testicular cancer survivors treated with platinum-based chemotherapy compared to the general population. It is associated with subclinical, unfavourable changes in intima-media thickness and baroreflex sensitivity, which may precede overt cardiovascular disease. The early development of overweight and dyslipidemia advocates the development of guidelines on detection and treatment from the start of chemotherapy and during follow-up. In addition, close collaboration with primary care physicians seems necessary with respect to extended follow-up and treatment of cardiovascular risk factors. Low testosterone and its association with central obesity appear to play a central role in the development of the metabolic syndrome and is a target for future intervention studies.

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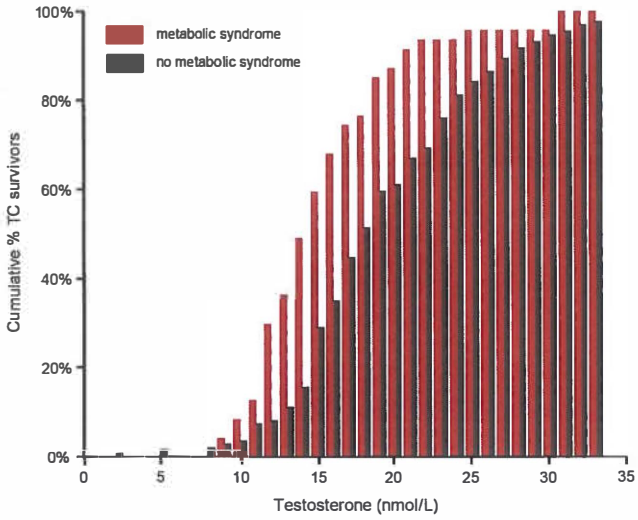
SUPPLEMENTARY FIGURES AND TABLES

		Study I (n = 370)	Study II (n = 173)
Baseline characteristics			
Age at start chemotherapy (years)	Median (range)	28 (16-64)	28 (16-52)
Chemotherapy regimen*			
BEP/EP	n (%)	262 (71%)	159 (92%)
PVB		27 (7%)	0 (0%)
PVB+		42 (11%)	0 (0%)
PVB/BEP		15 (4%)	1 (1%)
Other		24 (7%)	13 (7%)
Follow-up data			
Follow-up duration (years)	Median (range)	12 (3-29)	5 (3-20)
Age at end follow-up (years)	Median (range)	42 (19-73)	37 (19-59)
Deceased	n (%)	25 (7%)	not applicable
Death of testicular cancer		14 (4%)	-
Coronary heart disease (CHD)	n (%)	19 (5%)	not applicable
Age at CHD (years)	Median (range)	49 (30-62)	-
Follow-up duration at CHD (years)	Median (range)	15 (0-28)	-

Appendix table 1. Baseline characteristics and follow-up data of the testicular cancer patients in study I and the subpopulation included in study II.
(*) Chemotherapy regimen: BEP = bleomycin, etoposide, cisplatin; EP = etoposide, cisplatin; PVB = cisplatin, vinblastin, bleomycin; PVB+ = PVB followed by maintenance therapy with cisplatin and vinblastin; PVB/BEP = alternating courses of PVB and BEP; Other = CEB (carboplatin, etoposide, bleomycin) or BOP/VIP (bleomycin, vincristin, cisplatin / etoposide, ifosfamide, cisplatin)

Dependent variable	Odds ratio	95% CI	p-value
Metabolic syndrome	4.1	1.8-9.3	0.001
Central obesity	5.7	2.3-14.1	0.0001
High blood pressure	1.3	0.6-3.0	0.49
Low HDL cholesterol	1.1	0.5-2.4	0.80
High triglycerides	2.0	0.9-4.5	0.08
High glucose	2.9	1.1-7.5	0.03

Appendix table 2. Age-adjusted risk for the metabolic syndrome and its components in testicular cancer survivors with unsupplemented total testosterone levels <15 nmol/L (n=36). Risks are expressed as age-adjusted odds ratios compared to testicular cancer survivors with unsupplemented total testosterone levels ≥15 nmol/L (n=128) with 95% confidence interval (95%CI) and p-value (binary logistic regression). (Study II)



Appendix figure 1. Total testosterone level with respect to cumulative percentage of testicular cancer survivors with the metabolic syndrome and of testicular cancer survivors without the metabolic syndrome (excluding survivors with testosterone supplementation).

Genes and pathways associated with bleomycin- and cisplatin-induced endothelial damage: a preliminary report

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Chapter 8

ABSTRACT

Background

Chemotherapy-related endothelial damage contributes to the development of cardiovascular morbidity in testicular cancer patients. We aimed to obtain insight in relevant molecular events and search for candidate biomarkers of this endothelial damage.

Methods and materials

Human microvascular endothelial cells (HMEC-1) were exposed to bleomycin or cisplatin (IC_{10}, IC_{50}, IC_{90}), with untreated samples as control. Custom-made 18K cDNA microarrays were used, and read with an Affymetrix GMS428 scanner. Gene expression differences were analysed at the single gene level and in gene sets clustered in biological pathways. Validation was performed by qRT-PCR. A specific ELISA was used to measure protein levels of a candidate biomarker in testicular cancer patient plasma, collected before, during and after bleomycin-etoposide-cisplatin (BEP) chemotherapy.

Results

Expression data for 16k genes were available. Several genes were significantly differentially expressed in more than one experimental setting, e.g. *GDF15*, *ATF3* and *AREG*. Enriched gene sets included the 'P53 signaling' and 'Type I Diabetes Mellitus' pathways. qRT-PCR for *GDF15*, *ATF3* and *AREG* confirmed the cDNA microarray findings. In testicular cancer patients, GDF15 plasma levels increased during and after BEP-chemotherapy.

Conclusions

In this cDNA microarray analysis in HMEC-1 exposed to bleomycin or cisplatin, expression changed in single genes, e.g. *GDF15*, *ATF3* and *AREG*, and in biological pathways involved in cell death and inflammation. The observed changes in plasma GDF15-protein levels in testicular cancer patients indicate that this pre-clinical approach can guide the search for relevant biomarkers for endothelial damage. Further *in vitro* and *in vivo* exploration of vascular pathobiology and conformation is warranted. This strategy may facilitate the rationale towards selection of targets for intervention, and the introduction of early biomarkers for chemotherapy-related endothelial damage.

INTRODUCTION

The introduction of platinum-based chemotherapy in the late seventies has resulted in high cure rates in patients with metastatic testicular cancer. However, this chemotherapy coincides with side-effects, resulting in morbidity in successfully treated testicular cancer patients.[2] Relevant issues are the increased risks for second malignancies and cardiovascular disease (CVD).[7,12-14] CVD can arise during or shortly after treatment,[9,102] as well as in the long term.[12-14,16,17,36,103]

One of the mechanisms considered responsible for treatment-related cardiovascular complications is direct endothelial damage. In addition to induction of endothelial cell death,[22-24] both bleomycin[24,26,172] and cisplatin[24,25,27,175] indirectly influence endothelial cell function, e.g. through interference with inflammatory and fibrinolytic factors. Ultimately, this chemotherapy-induced cellular activation can progress to endothelial dysfunction, accelerated atherosclerosis and overt CVD.

A potentially useful approach to unravel factors involved in chemotherapy-related endothelial damage is large-scale gene expression profiling. With gene or cDNA microarray analysis, expression-differences upon exposure to a stimulus can be measured. One of the strengths of this approach is that genes with altered expression can be identified without *a priori* assumptions.

Several methodological approaches can be used to analyse gene expression data. In the conventional method of analyzing microarray-data the top few individual genes that are highly differentially expressed between phenotypes are reviewed. Such individual genes can be relevant. In addition, it is increasingly realized that small but coordinated changes in a set of genes clustered in a single pathway can have significant biological relevance. Integrating genes into functional sets enables consideration of all the available genomic information, rather than only the genes passing a certain significance threshold.

In this report we investigated the potential of cDNA microarray analysis to identify biological mechanisms underlying the development of chemotherapy-related endothelial damage. We studied the effects of bleomycin and cisplatin on the human microvascular endothelial cell line (HMEC-1) cell with cDNA gene expression profiling. To elucidate biological circuits that underlie endothelial cell responses, we not only studied the effects on single genes but also on clusters of gene sets involved in cellular pathways. Thereafter, changes in protein expression of a selected candidate gene were studied in testicular cancer patient plasma during and after bleomycin-etoposide-cisplatin (BEP) chemotherapy.

MATERIALS AND METHODS

Cell line model

HMEC-1 is an immortalized human dermal microvascular endothelial cell line that retains its morphologic and functional endothelial cell characteristics during several passages.[176] Cells were grown as a monolayer in MCDB-131 medium (Invitrogen, Merelbeke Belgium) supplemented with 10% Fetal Calf Serum (Bodinco, Alkmaar, the Netherlands), 10 mM L-glutamine (Invitrogen, Merelbeke, Belgium), 1 µg/mL hydrocortisone (Sigma-Aldrich, Amsterdam, the Netherlands) and 10 ng/mL human Epidermal Growth Factor (R&D Systems, Abingdon, UK), and were cultured at 37°C in a humidified atmosphere containing 5% CO_2. Experiments were performed between passages 15-30.

In an acute exposure model, HMEC-1 were left untreated as controls, or were treated with 0.3 (IC_{50}) or 1.5 µg/mL (IC_{90}) bleomycin and 2.6 (IC_{50}) or 12.9 µM (IC_{90}) cisplatin for 6, 24, and 48 hours (Figure 1A). In addition, in a chronic exposure model, lower doses were administered (IC10; bleomycin 0.06 µg/mL or cisplatin 0.52 µM,) twice weekly; cells were collected for analysis at day 30 (Figure 1B). Administration of cisplatin had to be withheld at the 7th administration because of considerable cell death. Bleomycin could be administered without interruption.

cDNA microarray experiments

Total RNA was isolated from the HMEC-1 cell lines by a RNeasy kit (Qiagen) and pooled for each time-point and drug from 2 independent experiments. After purification (Qiaquick PCR purification kit, Qiagen), amplified RNA (cRNA) samples were transformed to cDNA with reverse transcriptase, independently labeled with Cy3 (green) and Cy5 (red), randomized and hybridized to the custom-made 18K cDNA microarrays. Fluorescent images of the microarray slides were obtained with the Affymetrix GMS428 scanner (Affymetrix, Santa Clara, CA) for both fluorophores, signal intensities for each spot were quantified by dedicated IMAGENE 5.6 software (Biodiscovery, Marina del Rey, CA).

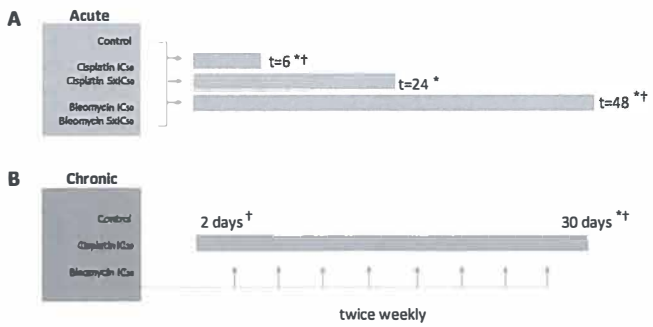

Figure 1. Experimental design: immortalized human dermal microvascular endothelial cells (HMEC-1) were exposed to bleomycin [0.3 (IC_{50}), 1.5 µg/mL (IC_{90})] or cisplatin [2.6 (IC_{50}), 12.9 µM (IC_{90})] for 6, 24 and 48 hours (acute exposure, A). Additionally, over the course of 30 days HMEC-1 was exposed to 0.06 µg/mL bleomycin (IC_{10}) or 0.52 µM cisplatin (IC_{10}) twice weekly (chronic exposure, B). In both experiments untreated samples served as controls. *(*) RNA-isolation and cDNA microarray experiments; (†) RNA-isolation and qRT-PCR*

Quantile normalization was applied to log2 transformed Cy3 and Cy5 intensities. Operon v2.0 (Human Genome Oligo Set V2) probe identifiers were converted to official gene symbols. Expression values of multiple probes targeting a single gene were averaged, resulting in a total of 15,950 distinct genes. Subsequently, expression data obtained from multiple hybridizations (n=4) of the same HMEC-1 specimen were averaged.

Class comparison

For class comparison in-house software was used. Analyses for samples exposed to cisplatin and bleomycin were performed in the same manner.

In the acute exposure setting, differentially expressed genes between HMEC-1 untreated samples and samples exposed to the different drug dosages (i.e. IC_{50} and $5xIC_{50}$) were tested with the non-parametric Cuzick test for linear trend, resulting in a Z-score and a P-value. This test was done for cells collected after 6 (t=6), 24 (t=24) and 48 (t=48) hours. For all three time-

points the Z-score resulting from the Cuzick tests was summed (i.e. $\Sigma Z = Z_{t=6} + Z_{t=24} + Z_{t=48}$); thereby selecting a constraint on genes that changed in a consistent direction with increasing concentrations over time. Genes were ranked according to their ΣZ-score.
In the chronic exposure model, a t-test was performed on gene expression levels obtained from samples exposed to the drugs (IC10 cisplatin or bleomycin) versus the untreated control samples that were collected after 30 days incubation. Results of these genes were ranked according to P-value.

Gene Set Enrichment Analysis
Gene Set Enrichment Analysis (GSEA)[177] was executed with GSEA 2.0 software package (Broad Institute of MIT and Harvard, Cambridge, MA). Expression data of all 15,950 genes were compared against functional gene sets to determine whether any of these sets were enriched in HMEC-1 treated with cisplatin or bleomycin in the acute as well as the chronic exposure model. The comparison was performed using 169 gene sets from Kyoto Encyclopedia of Genes and Genomes database (KEGG; http://www.genome.jp/kegg/). Statistical significance of enrichment was determined using an empirical gene-based permutation test using 1000 permutations. A false discovery rate (FDR) was calculated for each functional gene set, which represent the estimated probability that a given enrichment score represents a false positive finding. We report gene sets with a FDR ≤0.10 and a P-value ≤0.025. This FDR implies that an experiment is valid at least 9 out of 10 times.

Quantitative Real Time PCR
Next, differential gene expression from three genes found in the single gene analysis was evaluated by quantitative Real Time PCR (qRT-PCR). For this purpose, 12 RNA samples included in the cDNA microarray analysis were used (Figure 1A and 1B). Samples were DNAse treated to eliminate genomic DNA-contamination, and subsequently, RNA was reverse transcribed into cDNA. qRT-PCR was performed using Applied Biosystems TaqMan assays, according to the manufacturers protocol. Master Mix, primers and TaqMan probes were purchased from Applied Biosystems (Nieuwerkerk a/d IJssel, the Netherlands) The genes and their respective assay numbers were Growth Differentiation Factor 15 (*GDF15*; Hs 00171132_m1), Activating Transcription Factor 3 (*ATF3*; Hs 00231069_m1), Amphiregulin (*AREG*; Hs 00155832_m1) and of the housekeeping gene Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*; Hs 02758991_g1). All experiments were performed in triplicate using ABI PRISM® 7900 HT Sequence Detection System, with the following cycling conditions: 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 sec at 95 °C and 1 min at 60 °C. Relative quantity of target genes was calculated by dividing the mean cycle threshold (CT) for the gene of interest by the mean CT-value for the housekeeping gene GAPDH. Thereafter, expression of the untreated sample at t=6 or d2 was set at 1.00, and relative expression-differences were calculated.

GDF15 protein levels in plasma of testicular cancer patients during and after BEP-chemotherapy
From a prospective study on early chemotherapy-related cardiovascular changes in 41 testicular cancer patients treated with bleomycin- and cisplatin-based regimens, EDTA plasma was serially collected and stored in -20°C until analysis. The local medical ethics committee approved this study, and informed consent was obtained from all participants. Following

orchidectomy, all patients received, depending on their International Germ Cell Cancer Collaborative Group (IGCCCG) prognosis group, three or four three-weekly courses of BEP-chemotherapy [bleomycin (30 USP; days 2, 8, 15), etoposide (100 mg/m^2; days 1-5) and cisplatin (20 mg/m^2; days 1-5)]. During the first 6 days of each course patients were hydrated with 4 L NaCl 0.9%/day and received daily anti-emetic therapy with dexamethason and ondansetron. Blood samples were drawn at day 1, 8 and 15 of the first chemotherapy course, day 1 and 8 of the second and third course, [c1d1 (= baseline), c1d8, c1d15, c2d1, etc.], one month after completion and one year after start of chemotherapy. Reference data were obtained from healthy male siblings of adult childhood cancer survivors, who had participated as control subjects in a cross-sectional study on late cardiovascular sequelae of treatment for childhood cancer. Out of these healthy male siblings, a control group was selected with a comparable median age as the testicular cancer patients. Measurements in the controls were performed as described above.

Plasma GDF15-levels were determined by sandwich enzyme-linked immunosorbent assay (ELISA) with a commercially available kit (R&D Systems, Abingdon, UK). Furthermore, these GDF15 levels were related to plasma markers for endothelial activation [von Willebrand Factor (vWF), measured as described earlier][8] and systemic inflammation [high-sensitivity C-Reactive Protein (hsCRP), as described earlier][21].

For analysis of changes in plasma GDF15-levels, SPSS software package version 16.0 (SPSS Inc., Chicago, IL) was used. Non-normally distributed data were represented as median (range). For comparisons between groups the non-parametric Mann-Whitney U test or Kruskal-Wallis test was applied, as appropriate. The Wilcoxon's signed rank test was used for intra-individual changes in GDF15. Two-sided P-values ≤0.05 were considered significant.

RESULTS

cDNA microarray

Class comparison

The top 50 of most differentially expressed genes in the acute and chronic exposure models for bleomycin and cisplatin are summarised in Table 1. Figure 2 shows overlapping genes in the top 50s of the different models. From this analysis three candidate genes were selected for further exploration, e.g. GDF15, ATF3 and AREG.

BLEOMYCIN – ACUTE			BLEOMYCIN – CHRONIC			CISPLATIN – ACUTE			CISPLATIN – CHRONIC		
Gene		ΣZ	Gene		P-value	Gene		ΣZ	Gene		P-value
ATF3 *§	↑	9.574	CYP1B1	↑	<0.0001	CORT	↑	9.577	MX1 †	↓	<0.0001
KIAA1370	↑	9.574	KLF12	↑	<0.0001	TREM2	↑	9.222	GPR87	↓	<0.0001
RRAD	↑	9.552	PRSS35 §	↑	<0.001	ATF3 *‡	↑	9.039	IFI27	↓	<0.0001
C12orf5	↑	9.476	GL52	↓	<0.001	AVPI1	↑	8.757	JMJD4	↑	<0.001
GDF15 §	↑	9.454	ENPP4	↑	<0.001	AK1 *	↑	8.658	FAM15A	↓	<0.001
GPR87	↑	9.454	ZMAT1	↑	<0.001	VASN	↑	8.653	OAS3	↓	<0.001
MDM2	↑	9.356	TMEM184C	↑	<0.001	SESN2 *	↑	8.633	MX2	↓	<0.001
SESN2 *	↑	9.258	ADAM12 †	↑	<0.001	LRDD	↑	8.633	HLA-F	↓	<0.001
COL7A1	↑	9.16	RAB11FIP1	↑	<0.001	AREG ‡	↑	8.633	AREG †‡	↓	<0.001
ATG16L2	↑	9.16	PTGER2	↑	<0.001	PLCD1	↑	8.586	LOC1576	↑	<0.001
AK1 *	↑	9.16	SERPINB2	↑	<0.001	ADM	↑	8.549	ALKBH8	↑	<0.001

Table 1 – continued

BLEOMYCIN – ACUTE			BLEOMYCIN – CHRONIC			CISPLATIN – ACUTE			CISPLATIN – CHRONIC		
Gene		ΣZ	Gene		P-value	Gene		ΣZ	Gene		P-value
FAS	↑	8.964	SCFD2	↑	<0.01	LRRTM2	↑	8.524	ABCC4	↑	<0.001
LIF	↑	8.964	KRTAP4-8	↑	<0.01	DEDD2	↑	8.457	HIST2BE	↓	<0.001
VWCE	↑	8.866	C10orf136	↑	<0.01	RALGDS	↑	8.37	RFT1	↑	<0.001
TP53INP1	↑	8.817	HES1 †	↓	<0.01	DNAJB2	↑	8.364	LRRC38	↑	<0.001
VDR	↑	8.811	COL1A2	↑	<0.01	MSX1	↑	8.328	SNAI1 †	↓	<0.001
C4orf18	↑	8.768	HIST1H2BJ	↑	<0.01	MST150	↑	8.322	C19orf42	↑	<0.01
FERMT1	↑	8.734	RBPJL	↑	<0.01	NR4A3	↑	8.157	LOC1171	↓	<0.01
FUCA1	↑	8.691	CRTAC1	↓	<0.01	FDXR	↑	8.143	SIAE	↓	<0.01
MCC	↑	8.691	CCDC148 †	↓	<0.01	ITPKA	↑	8.143	IQGAP2	↓	<0.01
NELF	↑	8.691	ROR1	↑	<0.01	KREMEN2 ‡	↑	8.143	POLA1	↑	<0.01
BTG2	↑	8.691	EDN1	↓	<0.01	SLC31A2	↑	8.126	PDK4	↓	<0.01
TGFBR1	↑	8.691	TGFB2 §	↑	<0.01	CEACAM1	↑	8.107	ATF3 †‡	↓	<0.01
TMEM131	↑	8.572	PRKCZ	↑	<0.01	IRF5	↑	8.101	TNFSF10	↓	<0.01
CDH10	↑	8.572	MARVELD2	↑	<0.01	FOXL2	↑	8.101	DHX37	↑	<0.01
FZD2	↓	-7.775	ATF3 †§	↓	<0.01	VCAM1 *	↓	-8.432	TOM4L	↑	<0.01
C3orf36	↓	-7.809	CD82	↓	<0.01	C7orf10	↓	-8.437	IFIT3	↓	<0.01
TRIB2 *	↓	-7.817	XTP3TPA	↑	<0.01	C3orf26	↓	-8.438	IFI44L	↓	<0.01
SMA5	↓	-7.835	SPTAN1	↓	<0.01	MYRIP	↓	-8.535	TNC †	↑	<0.01
NDRG4	↓	-7.869	KIAA1655	↑	<0.01	ROR1	↓	-8.56	KREMEN2‡	↓	<0.01
EBPL	↓	-7.873	DDB2	↓	<0.01	QKI	↓	-8.597	HNMT	↓	<0.01
TGFB2 §	↓	-7.912	SNAI1 †	↓	<0.01	TRIB2 *	↓	-8.628	BRD9	↑	<0.01
MYCN	↓	-7.971	STC2	↑	<0.01	CDKAL1	↓	-8.647	BEX2	↓	<0.01
SEMA3A *	↓	-7.971	TNC †	↑	<0.01	TFPI	↓	-8.658	MGC33894	↓	<0.01
GIMAP2	↓	-7.992	LRRIQ1	↓	<0.01	LTBP1	↓	-8.664	DUSP1 †	↓	<0.01
ZFP36	↓	-8.001	ASMT	↑	<0.01	PLK1	↓	-8.686	C7orf54	↓	<0.01
UBE2G2	↓	-8.044	GATA6	↑	<0.01	NAV1	↓	-8.714	ID4	↑	<0.01
RNASE1	↓	-8.069	GDF15 †§	↓	<0.01	PAPPA	↓	-8.726	CCDC148 †	↓	<0.01
MXD3	↓	-8.21	PCDHGA3	↓	<0.01	PIF1 *	↓	-8.765	MDM2	↓	<0.01
HIST12AM	↓	-8.218	DDX19A	↑	<0.01	DHRSX	↓	-8.891	PRAP1	↓	<0.01
C14orf94	↓	-8.222	RARB	↑	<0.01	GMDS	↓	-8.928	FOS	↓	<0.01
PIF1 *	↓	-8.261	CCND2	↓	<0.01	LAMA4	↓	-9.02	HES1 †	↓	<0.01
OASL	↓	-8.265	BCS1L	↑	<0.01	DKFZP0335	↓	-9.02	ASPA	↓	<0.01
PRSS35 §	↓	-8.44	BIRC7	↑	<0.01	CD9	↓	-9.051	GDF15 †	↓	<0.01
CLEC14A *	↓	-8.482	MX1 †	↓	<0.01	FAT4	↓	-9.124	COL9A3	↓	<0.01
HOXD8	↓	-8.679	RERG	↑	<0.01	DOCK1	↓	-9.222	LGALS9	↓	<0.01
HIST1H1D	↓	-8.713	ADAMTS12	↑	<0.01	SMAD7	↓	-9.32	NR4A1	↓	<0.01
CLEC3B	↓	-8.866	DUSP1 †	↓	<0.01	FHOD3	↓	-9.381	ADAM12 †	↑	<0.01
VCAM1 *	↓	-9.16	AREG †	↓	<0.01	CLEC14A *	↓	-9.418	RNASE7	↓	<0.01
C9orf3	↓	-9.552	OSBPL1A	↑	<0.01	SEMA3A *	↓	-9.418	HHATL	↓	<0.01

Table 1. Top 50 genes with largest difference in expression in the different exposure settings in HMEC-1.
(*) Overlapping genes in the acute exposure model for both drugs; (†) Overlapping genes in the chronic exposure model for both drugs; (‡) Overlapping genes in the acute and chronic exposure models for cisplatin; (§) Overlapping genes in the acute and chronic exposure models for bleomycin.

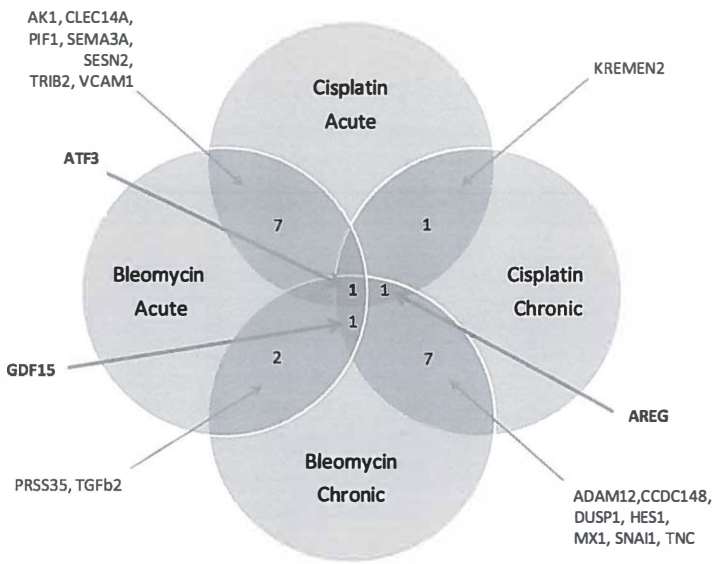

Figure 2. Results from the class comparison analysis: overlapping genes from top 50's of most differentially expressed genes in HMEC-1, resulting from acute and chronic exposure to bleomycin and cisplatin.

cDNA microarray – GSEA

Pathways enriched at a FDR ≤0.10 and P-value ≤0.025 in the GSEA are summarised in Table 2. In the acute exposure model to bleomycin, six pathways were enriched (all upregulated), while no pathways were enriched in the chronic setting with the set criteria for FDR. For cisplatin, 12 pathways were enriched in the acute exposure model (upregulated n=3, downregulated n=9) while six pathways were enriched in the chronic exposure model (all downregulated).

Both the 'p53-pathway' and the 'Type I Diabetes Mellitus' gene sets were upregulated in acute exposure to bleomycin and cisplatin, and downregulated in chronic exposure to cisplatin. Genes included in this gene set are summarised in Appendix I.

qRT-PCR

To validate changes in expression of *GDF15*, *ATF3* and *AREG* we performed qRT-PCR. In the acute exposure model, mRNA-expression of all three genes decreased in untreated cells at t=48 compared to t=6 (Figure 3A). Contrarily, in cells exposed to bleomycin or cisplatin, mRNA-expression increased at t=48 compared to t=6, except for *ATF3* after exposure to bleomycin. At t=48 mRNA-expression of all three genes in treated cells was higher compared to untreated cells at the same time-point.

In the chronic exposure model, mRNA-expression of *GDF15*, *ATF3* and *AREG* increased in untreated cells at day 30 compared to day 2 (Figure 3B). In cells exposed to bleomycin or cisplatin these increases were also observed, with the exception of *AREG*-expression in cisplatin-treated cells. Compared to untreated cells, mRNA-expression of all genes in treated cells at day 2 and day 30 was higher. Overall, this qRT-PCR confirms that exposure to bleomycin or cisplatin induces changes in gene expression in *GDF15*, *ATF3* and *AREG* in HMEC-1.

Gene Set Enrichment Analysis	BLEOMYCIN – ACUTE			CISPLATIN – ACUTE			CISPLATIN-CHRONIC		
		FDR	P-value		FDR	P-value		FDR	P-value
Cellular Processes; Cell Communication									
Adherens junction				↓	0.07	0.003			
Focal adhesion				↓	0.04	<0.0001			
Cellular Processes; Cell Growth and Death									
p53 signaling pathway	↑	0.001	<0.0001	↑	0.02	<0.0001	↓	0.1	<0.001
Environmental Information Processing; Signal Transduction									
TGF-beta signaling pathway				↓	0.05	<0.0001			
Genetic Information Processing; Folding. Sorting and Degradation									
Ubiquitin mediated proteolysis				↓	0.04	0.001			
Human Diseases									
Endometrial cancer				↓	0.04	<0.0001			
Cholera infection	↑	0.10	0.005						
Type I diabetes mellitus	↑	0.07	<0.0001	↑	0.05	<0.0001	↓	0.01	<0.001
Neurodegerative disease							↓	0.09	0.008
Prion diseases							↓	0.08	0.01
Metabolism; Carbohydrate Metabolism									
Butanoate metabolism	↑	0.02	<0.0001						
Glyoxylate and dicarboxylate metabolism				↓	0.09	0.02			
Reductive carboxylate cycle				↓	0.04	0.002			
Glycosylphosphatidylinositol(GPI)-anchor biosynthesis	↑	0.03	0.002						
N-Glycan biosynthesis				↓	0.08	<0.0001			
Linoleic acid metabolism	↑	0.08	0.005	↑	0.09	0.01			
Polyunsatyrated fatty acid biosynthesis				↓	0.04	0.002			
Organismal Systems; Immune System									
Antigen processing and presentation							↓	0.0	<0.001
Toll-like receptor signaling pathway							↓	0.08	<0.001

Table 2. Gene Set Enrichment Analysis on gene expression profiles from HMEC-1 in acute and chronic exposure to bleomycin and cisplatin, using pathway definitions from KEGG. No pathways were enriched according to these criteria in chronic exposure to bleomycin.

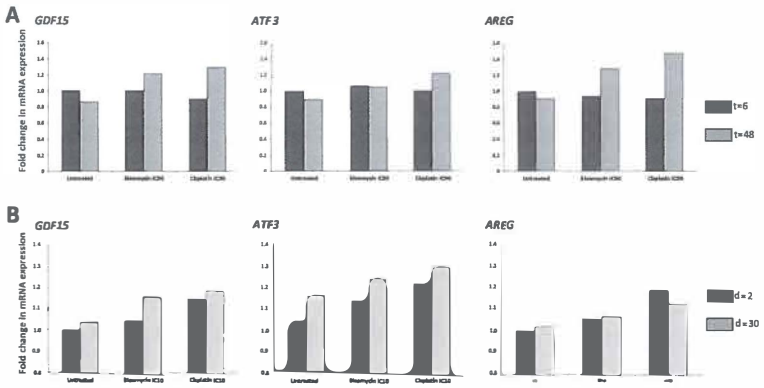

Figure 3. Changes in mRNA-expression of three genes from the top 50 of most differentially expressed genes in the class comparison analysis in acute (A) and chronic (B) exposure to bleomycin or cisplatin. The fold increase in expression of each gene is relative to mRNA-expression in untreated cells at t=6 or d=2. Abbreviations: Growth-Differentiation Factor 15 (*GDF15*), Activating Transcription Factor 3 (*ATF3*), Amphiregulin (*AREG*).

Plasma GDF15-levels in testicular cancer patients treated with BEP-chemotherapy

Based on differences in gene expression in the cDNA microarray analysis and qRT-PCR, we measured plasma protein levels of GDF15 during BEP-chemotherapy in testicular cancer patients. Baseline (i.e. pre-chemotherapy) GDF15-levels in testicular cancer patients were not different from healthy age-matched males (Figure 4A). Patients in the IGCCCG good prognosis group had lower baseline GDF15-levels than patients in intermediate or poor prognosis groups [good prognosis: median 362.9 pg/mL (range 197.6-1059.5; n=33), intermediate/poor prognosis: median 689.1 pg/mL (range 186.8-1935.0; n=8); P=0.04]. Pre-chemotherapy GDF15-levels were not related to age (rs= 0.08; P=0.61).

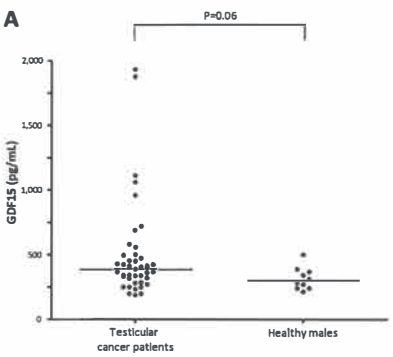

Figure 4A. Plasma levels of GDF15 in patients with metastatic testicular cancer prior to start of BEP-chemotherapy, compared to healthy age-matched males.

During BEP-chemotherapy, GDF15-levels increased compared to baseline, with significantly higher levels 1 months and 1 year post-chemotherapy (Figure 4B, Table 3). Compared to pre-chemotherapy, plasma levels of vWF and hsCRP changed significantly during treatment (Table 3). After completion of chemotherapy, vWF-levels remained persistently elevated, whereas hsCRP returned to pre-chemotherapy values. At baseline, levels of GDF15 were related to

levels of vWF (r_s= 0.35; P=0.03) and hsCRP (r_s= 0.39; P=0.014). Throughout treatment, levels of GDF15 correlated with hsCRP at c1d8 (r_s= 0.44; P=0.01) and with vWF at c3d8 (r_s= 0.40; P=0.017). At the follow-up visit one month after completion of chemotherapy, levels of GDF15 and vWF were strongly correlated (r_s= 0.56; P=0.001), whereas this relation was not found for GDF15 and hsCRP (r_s= 0.18; P=0.28). One year after start of treatment, no relation between GDF15-levels and vWF or hsCRP (r_s= 0.28; P=0.12; r_s= 0.10; P=0.57) was found.

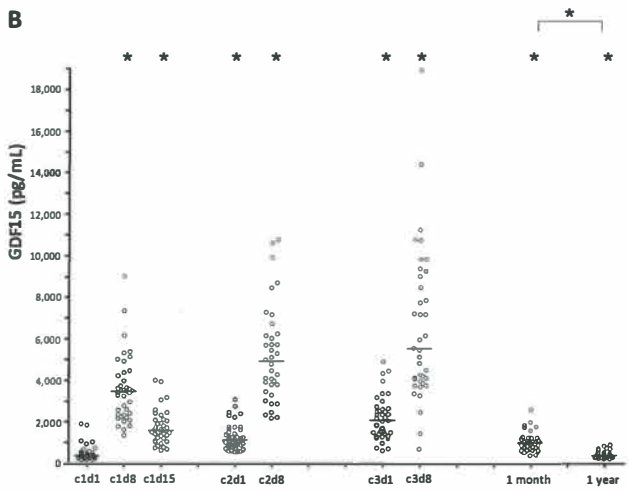

Figure 4B. Changes in plasma protein GDF15-levels before, during and after completion of BEP-chemotherapy for testicular cancer. The sample at c1d1 is drawn before initiation of chemotherapy. (*) p<0.05 compared to baseline value or indicated time-point (Wilcoxon signed rank test.

	GDF15 (pg/mL)		vWF (%)		hsCRP (mg/L)	
	Median	Range	Median	Range	Median	Range
Course 1 day 1 (=baseline)	383.1	186.8-1935.0	100	42-297	2.0	0.2-87.1
Course 1 day 8	3473.7 *	1344.5-9028.3	164 *	56-319	0.6 *	0.2-4.9
Course 1 day 15	1587.2 *	655.2-4014.9	145 *	57-394	4.2 †	1.1-101.0
Course 2 day 1	1145.5 *	593.6-3074.5	143 *	34-360	5.1	1.1-39.8
Course 2 day 8	4898.0 *	2183.0-10794.9	197 *	66-464	0.6 *	0.2-21.1
Course 3 day 1	2067.7 *	639.0-4918.0	194 *	66-440	3.7	0.3-39.9
Course 3 day 8	5542.8 *	702.3-18958.9	197 *	81-419	0.6	0.2-25.7
One month	1009.6 *	409.7-4737.1	135 *	56-249	2.2	0.4-29.1
One year	395.2 *‡	246.9-913.2	115 *‡	49-218	1.5	0.2-14.3

Table 3. Levels of Growth Differentiation Factor 15 (GDF, pg/mL), von Willebrand Factor (vWF, %) and high-sensitivity C-Reactive Protein (hsCRP, mg/L) before, during and after completion of bleomycin- and cisplatin-based chemotherapy for testicular cancer.

(*) P<0.01 compared to baseline, Wilcoxon Signed Rank test; (†) P<0.05 compared to baseline, Wilcoxon Signed Rank test; (‡) P<0.01 compared to one month after completion of chemotherapy, Wilcoxon Signed Rank test; (§) P<0.05 compared to one month after completion of chemotherapy, Wilcoxon Signed Rank test

DISCUSSION

This study shows that acute and chronic exposure to bleomycin and cisplatin induces changes in gene expression in HMEC-1, as measured with cDNA microarray analysis. We found several single genes with significant changes in expression upon exposure to these drugs, i.e. *GDF15*, *ATF3* and *AREG*. In addition, GSEA revealed clusters of genes involved in apoptotic and inflammatory pathways that were affected in this model. Furthermore, we showed for one of the relevant genes that BEP-chemotherapy did indeed affect plasma protein levels of GDF15 in testicular cancer patients, apparent from increases during chemotherapy. Taken together, these experiments show that cDNA microarray studies in this endothelial cell model are a useful strategy to explore mechanisms related to and select candidate biomarkers involved in chemotherapy-related endothelial damage.

cDNA microarray analysis provides information on changes in single genes as well as in clusters of genes involved in biological pathways, affected by exposure to a pathogenic stimulus like chemotherapeutic drugs. This analysis permits the evaluation of effects on various relevant cell types, e.g. tumour cells or endothelial cells. The advantage of this method is that changes in gene expression are assessed without a priori assumptions, rather than using a candidate-based approach based on data obtained from the literature. This permits the finding of unknown genes and pathways related to the development of drug-related vascular damage. In our class comparison analysis for the acute exposure model, a constraint was put on genes that had a changed RNA expression in a consistent direction by using the sum of the linear tests for trend (ΣZ). Hereby, genes are selected with changes in RNA expression in a consistent direction with increasing concentrations over time (i.e. untreated, IC_{50}, IC_{90} after 6, 24 and 48 hours). In the chronic exposure model, changes in RNA expression were estimated by calculating differences between cells exposed to lower concentrations (IC_{10}) of cytostatics over 30 days compared to untreated cells, as the time-course of the experiment by itself may also change RNA-expression. With these methods used for class comparison, expression of several genes changed significantly after exposition to bleomycin or cisplatin, with a number of overlapping genes in different experimental settings, i.e. *GDF15*, *ATF3* and *AREG*.

Next to exploring differences in expression of individual genes as biomarkers for chemotherapy-related endothelial damage, we aimed to analyze smaller but coordinated changes in sets of genes involved in biological pathways.[177] Using GSEA, a powerful and robust tool for comparison of RNA expression, differences were compared against functional gene sets that are defined in the KEGG-database. In this analysis, several gene sets were significantly enriched at the set criteria for FDR, including 'p53 pathway' and 'Type I Diabetes Mellitus' in acute exposure to bleomycin and cisplatin, and chronic exposure to cisplatin. The finding that p53-related genes were affected by cisplatin as well as bleomycin illustrated the validity of our approach, as both drugs excert their key therapeutic effect by cellular apoptosis induction. The enriched 'Type I Diabetes Mellitus' gene set includes several genes involved in inflammatory processes, e.g. HLA-molecules, interleukins and TNF, indicating that bleomycin and cisplatin induce an inflammatory response in endothelial cells. This finding is completely in line with studies in testicular cancer patients treated with cisplatin-based regimens, which showed higher rates of systemic inflammation and endothelial dysfunction.[8,20,21] As circulating platinum remains detectable in the circulation years to decades after cisplatin treatment,[28] long-term testicular cancer survivors may well have ongoing vascular damage and chronic low-grade endothelial inflammation. When chronic inflammatory responses prove to be an important

pathogenic factor for the development of chemotherapy-induced endothelial damage, intervention with anti-inflammatory drugs is a rationale approach to alleviate these effects.

Based on our microarray findings we studied plasma protein levels of GDF15 in testicular cancer patients before and during treatment with BEP-chemotherapy. The increases in GDF15 throughout treatment as well as post-chemotherapy may partly relate to cancer-related mechanisms, as GDF15 was higher in patients with more advanced disease stage. However, GDF15-levels correlated with proteins known to reflect chemotherapy-related endothelial damage in testicular cancer patients, vWF and hsCRP.[8,21] This supports the hypothesis that the increases in GDF15 may importantly relate to systemic endothelial damage.

The cytokine GDF15 is a member of the transforming growth factor β (TGFβ) family. Stimulation of several cell types, e.g. by Tumor Necrosis Factor α (TNFα), interleukins or pro-inflammatory cytokines results in increased GDF15-expression.[178] Release of GDF15 can induce anti-inflammatory, anti-apoptotic and anti-proliferative effects, thereby exerting vasculoprotective mechanisms. Therefore, increases in GDF15-plasma levels in our patient cohort may well result from increased production by endothelial cells and/or macrophages, to compensate for chemotherapy-related damage. In cardiovascular medicine, plasma levels of GDF15 are explored as a predictive marker for risk of death or other adverse events in patients presenting with symptomatic coronary events,[179,180] as well as for cardiovascular events in otherwise healthy subjects.[181] A number of other studies suggest that GDF15 plays a relevant role in cardiovascular damage.[182] In a study evaluating gene expression changes in prostate cancer samples pre- and post docetaxel/mitoxantrone treatment, GDF15 was one of highest upregulated genes post-treatment,[183] illustrating that cytostatics can influence GDF15-expression.

Further exploration of the role of ATF3 and AREG may provide more insight in mechanisms of chemotherapy-related endothelial damage. Data from the literature support the relevance of the gene ATF3 in this context. ATF3 is a downstream member of the MAP-kinase signalling pathway that encodes for a nuclear factor that stimulates transcription upon cellular stress. Fast upregulation of ATF3 is a central stress response in different endothelial cell models, induced by various noxious stimuli.[184-189] Interference with ATF3-levels protected cells from apoptosis induction, e.g. induced by TNFα in HUVEC,[190] by cisplatin in a human glioblastoma cell line,[185] or related to doxorubicin in cardiomyocytes.[191] Interestingly, increased ATF3-expression measured immunohistochemically was found in atherosclerotic areas of human iliac arteries.[186] Few studies addressed AREG, a member of the epidermal growth factors receptors that plays an important role in cellular proliferation and survival. Breast cancer cells exposed to cisplatin secreted the AREG-protein over extended periods of time, i.e. up to 72 hours after exposition.[192]

In conclusion, cDNA microarray analysis on HMEC-1 facilitates exploration of genes and pathways associated with cisplatin- and bleomycin-related endothelial damage. Several genes were found to be differentially expressed after exposition to these drugs, e.g. *GDF15*, *ATF3* and *AREG*. In GSEA, clusters of genes involved in cell death and inflammation were affected. The observed changes in plasma GDF15-protein levels in testicular cancer patients indicate that this pre-clinical approach can be translated to a clinical setting. Further in vitro and in vivo exploration is warranted. Eventually, this may facilitate the rationale towards selection of targets for intervention, and the introduction of early surrogate biomarkers for chemotherapy-related endothelial damage.

Acknowledgments

We thank Gert-Jan Meersma, Haukeline Volders and Nynke Zwart for their technical assistance and for collection of patient samples. Supported by Grant 2009-4365 from the Dutch Cancer Society.

p53 pathway	Type I Diabetes Mellitus pathway
APAF1	CD28
ATM	CD80
BAX	CD86
BCL2	CPE
CCND1	FAS
CCNE1	FASLG
CDK2	GAD1
CDK4	GAD2
CDKN1A	GZMB
E2F1	HLA-A
GADD45A	HLA-C
MDM2	HLA-DMA
PCNA	HLA-DMB
RB1	HLA-DOA
TIMP3	HLA-DOB
TP53	HLA-DPA1
	HLA-DPB1
	HLA-DQA1
	HLA-DQA2
	HLA-DQB1
	HLA-DRA
	HLA-DRB5
	HLA-E
	HLA-F
	HLA-G
	ICA1
	IFNG
	IL12A
	IL12B
	IL1A
	IL1B
	IL2
	INS
	LTA
	PRF1
	PTPRN
	PTPRN2
	TNF

Appendix table 1. Genes included in the 'p53 pathway' and 'Type I Diabetes Mellitus pathway' gene sets in the KEGG database, ranked in alphabetical order.

Summary, General Discussion & Future Perspectives

Chapter 9

BACKGROUND

Since the introduction of platinum-based chemotherapy regimens in the late 1970's,[4] metastatic testicular cancer has become a highly curable malignancy with a 10-year survival rate of around 80%.[2,5] As a result of this favourable prognosis and together with a still increasing incidence,[1,5] the population of successfully treated testicular cancer survivors is steadily growing. Because of a normal life-expectancy when cured from testicular cancer, the risk for treatment-related complications in these patients is increasingly recognized.[2,6] Examples of these side-effects are infertility, neurotoxicity, second malignancies and cardiovascular disease (CVD).[6,7]

Acute cardiovascular complications during or shortly after completion of chemotherapy can manifest as arterial or venous thrombo-embolic events.[8,9] Moreover, the development of Raynaud's phenomenon[10] and the occurrence of sometimes even fatal pulmonary toxicity[11] cause unwanted side-effects of this curative treatment. In addition to early events, testicular cancer survivors have an increased risk for late CVD[16,193] and subclinical signs for atherosclerosis.[18-21]

Both clinical[8] and pre-clinical[22-27] studies have shown that direct chemotherapy-related endothelial damage is an important mechanism for the development of these vascular complications. Furthermore, cardiovascular risk factors and endocrine disturbances can contribute to cardiovascular morbidity observed in long-term testicular cancer survivors.[31]

Early recognition and possibly prevention of these treatment-related complications is needed to conserve an optimal health condition of testicular cancer survivors. The development of CVD is a gradual process, and early interventions may slow down or stop the progression towards overt clinical morbidity. Biomarkers for treatment-related cardiovascular damage can identify patients at increased risk for CVD, and guide treatment and follow-up schedules. In addition, specific biomarkers can increase insight in the pathogenesis of vascular complications, and point to possible targets for intervention. Furthermore, such biomarkers may be used as surrogate markers for cardiovascular disease, and thereby facilitate intervention studies that are aimed to decrease the incidence of chemotherapy-related CVD.

SUMMARY

The studies described in this thesis aim to search for early biomarkers of subclinical treatment-related cardiovascular damage in testicular cancer patients treated with cisplatin-based chemotherapy.

Up to now, the most commonly used method for evaluation of cancer treatment-related cardiovascular damage is measurement of the Left Ventricular Ejection Fraction (LVEF). However, the LVEF may underestimate actual cardiac damage as subclinical changes in cardiac function can occur with a preserved systolic function. In **Chapter 2** we summarised advantages, disadvantages, and the Level of Evidence[35] for the use of different methods. This literature review confirmed that current monitoring strategies for detection of cardiovascular damage are mainly based on moderate evidence. Therefore, studies are needed that explore reliable and sensitive monitoring strategies for early detection of cancer treatment-related cardiovascular damage.

We prospectively measured changes in a comprehensive panel of biomarkers involved in various aspects of vascular pathobiology, e.g. circulating endothelial cells (CECs) in blood and plasma biomarkers for endothelial activation, inflammation and pro-thrombotic state in testicular cancer patients treated with bleomycin-etoposide-cisplatin (BEP) chemotherapy (**Chapter 3**). Within this panel of vascular biomarkers, changes in levels of CECs and vWF as well as increases in carotid IMT may point to testicular cancer patients with augmented endothelial damage. These biomarkers should be included in studies addressing the cisplatin chemotherapy-related damage phenotype in testicular cancer patients, consisting of accelerated atherosclerosis and cardiovascular morbidity. Extended follow-up studies will reveal the predictive value of early rises in CECs and persistent endothelial activation for late CVD in this patient cohort. Eventually, such a marker panel should identify patients in whom active intervention to alleviate or prevent chemotherapy-related CVD is indicated.

Long-term testicular cancer survivors have an increased incidence of cardiac dysfunction.[13,36] We prospectively measured early changes in cardiac function in testicular cancer patients by means of sensitive echocardiography parameters and serum levels of the cardiac biomarker N-Terminal pro Brain Natriuretic Peptide (NT-proBNP). At a median of 10 months after BEP-chemotherapy, diastolic function significantly declined and NT-proBNP-levels were higher than baseline (**Chapter 4**). Next, over a median follow-up of 7 years, the incidence of abnormal diastolic parameters significantly increased in this patient cohort (**Chapter 5**). Overall the rate of systolic dysfunction remained low, with one out of 37 patients (2.7%) with an abnormal LVEF. Changes in plasma levels of the cardiac biomarkers NT-proBNP, troponin and galectin-3 were not predictive for late cardiac abnormalities. At both post-chemotherapy cardiac evaluations, declines in diastolic function parameters were larger in the presence or development of hypertension and obesity. These associations stress the need to monitor and treat cardiovascular risk factors in testicular cancer survivors.

Carotid IMT is a measure for systemic atherosclerotic burden and an independent predictive marker for cardiovascular endpoints in the general population.[143] Accelerated increases in IMT may identify subjects with a higher risk for CVD. During a 7-year longitudinal follow-up of testicular cancer survivors treated with BEP-chemotherapy, carotid IMT significantly increased (**Chapter 6**). At 7 years their median IMT was not different from healthy males, whereas these survivors had a higher age-related yearly increase in IMT. The IMT progression rate was associated with increases in serum lipids, revealing a potential target for intervention. Patients who developed Raynaud's phenomenon after chemotherapy had a higher IMT during follow-up and a steeper age-related increase in IMT. This association suggests that Raynaud's phenomenon may be a surrogate clinical marker for chemotherapy-related endothelial damage, and patients who develop this phenomenon may be more prone to develop CVD.
The metabolic syndrome is a cluster of cardiovascular risk factors that are associated with a higher risk for CVD. We investigated the prevalence and associated features of this syndrome in a cross-sectional study in ≥3 year post-chemotherapy testicular cancer survivors. The incidence of the metabolic syndrome was 25%, which is 2-fold higher than in a healthy age-matched reference population (**Chapter 7**). Separate components of the metabolic syndrome developed relatively early; after a median number of follow-up years of 1.7 for obesity, 0.9 for hypercholesterolemia and 5.1 for hypertension. Presence of the metabolic syndrome was

associated with higher levels of systemic inflammation, a more pro-thrombotic state and higher carotid IMT. This early development and higher rate of metabolic syndrome emphasises the need for guidelines on early detection and treatment of these risk factors.

To obtain more insight in molecular events involved in chemotherapy-related endothelial damage, we performed a cDNA microarray analysis in human microvascular endothelial cells (HMEC-1) exposed to bleomycin or cisplatin. In this preliminary study, significant changes in expression occurred in single genes, e.g. *GDF15*, *ATF3* and *AREG*. Furthermore, biological pathways involved in cell death and inflammation were affected (**Chapter 8**). With qRT-PCR the differences in gene expression in *GDF15*, *ATF3* and *AREG* were validated. In addition, we measured changes in protein levels of the cytokine encoded for by the *GFD15*-gene. These protein levels increased in plasma of testicular cancer patients during treatment with BEP-chemotherapy. This study indicates that cDNA microarray analysis offers the road towards a translational approach to investigate and explore new candidate biomarkers and mechanisms for chemotherapy-related endothelial damage. Further *in vitro* and *in vivo* exploration is warranted. Eventually, this may facilitate the rationale towards targets for intervention, and the introduction of surrogate biomarkers for early vascular damage.

GENERAL DISCUSSION & FUTURE PERSPECTIVES

Strategies to decrease the development of cardiovascular complications in testicular cancer patients

Progression from endothelial dysfunction towards overt CVD is a gradual process, starting with early endothelial activation and followed by a silent progression over the course of years.[29,30] By detection of chemotherapy-related endothelial damage in an early and still reversible phase, those patients who have an increased risk for CVD can be identified.

Figure 1 shows a hypothetical scheme that summarises different strategies to decrease the rate of cardiovascular damage during and after a potentially toxic cancer treatment. The different lines represent the timing of vascular complications. First, patients may develop early signs of vascular damage and endothelial dysfunction during BEP-chemotherapy (line I). Furthermore, late endothelial dysfunction may arise during follow-up, i.e. years to decades after completion of treatment (line II). In addition, a group of patients have a normal, physiological decrease in endothelial function during ageing (line III). In the following section, aspects of the strategies to decrease cardiovascular complications (numbers 1 to 5 in Figure 1) will be discussed, and new insights from the studies described in this thesis will be addressed.

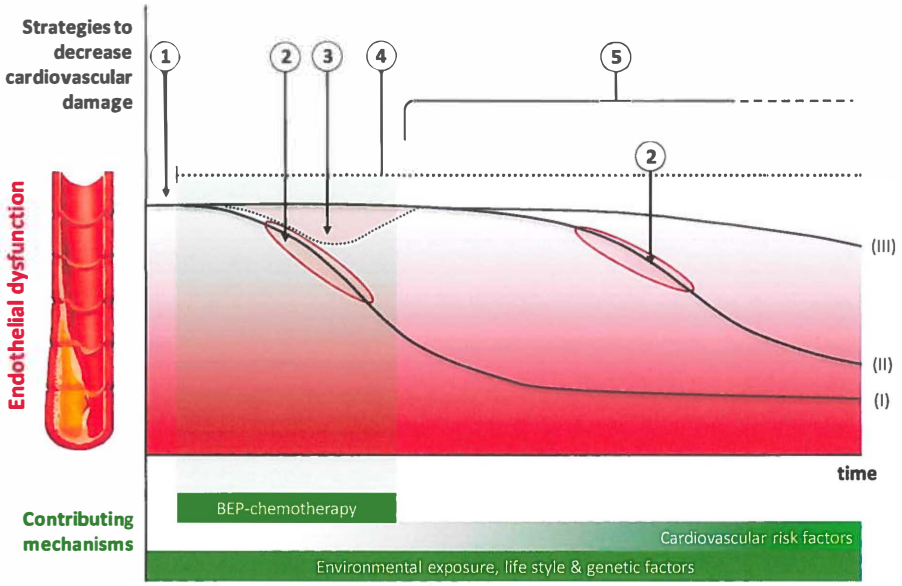

Figure 1. Hypothetical schematic representation of changes in endothelial status in testicular cancer patients treated with BEP-chemotherapy. (I) Early endothelial dysfunction; (II) Late endothelial dysfunction; (III) Physiological decrease in endothelial function during ageing. Green bars indicate factors that contribute to the development of endothelial dysfunction. Different strategies to reduce endothelial damage progression are indicated with numbers 1 to 5. (1) estimation of a baseline risk; (2) subclinical changes; (3) temporary phenomena; (4) intervention/prevention (5) follow-up schedules.

1. Estimation of a baseline risk for cardiovascular damage

Prior to initiation of BEP-chemotherapy, a baseline risk for treatment-related complications includes patient-related characteristics and risk factors for CVD. These consist of a medical history of CVD, presence of cardiovascular risk factors and risk behaviour such as smoking, a cholesterol-rich diet and sedentary life style. In our study on early changes in cardiac function after BEP-chemotherapy, a high pre-treatment blood pressure was associated with larger declines in diastolic parameters (Chapter 4). This finding stresses the relevance of cardiovascular risk factors already at this early stage.

A promising strategy in this regard is identification of specific genetic variations, i.e. Single Nucleotide Polymorphisms (SNPs), that modulate the susceptibility for treatment-related cardiovascular damage. Candidate SNPs relate to variations in pharmacokinetics and pharmacodynamics of cytostatics, a baseline tendency to develop CVD, and capacity for repair of treatment-related damage.6 Although some studies investigated candidate SNPs, up to now no firm relations between specific variations and the risk for cardiovascular complications have been established.[194,195,196]

2. Changes in biomarkers for subclinical cardiovascular damage

Biomarkers for endothelial activation

Plasma levels of endothelial activation markers increase during BEP-chemotherapy and remain persistently higher than baseline up to one year post-chemotherapy (Chapter 3). Furthermore,

during 7-year follow-up levels of soluble adhesion molecules increase compared to 11 months post-chemotherapy (Chapter 6). Taken together, data from these studies indicate that increases in markers for endothelial activation indicate subclinical vascular damage during and after treatment, which may mechanistically contribute to atherosclerosis.

Biomarkers for systemic inflammation

We observed significant changes in plasma levels of high-sensitivity C-reactive protein (hsCRP) during BEP-courses, whereas levels returned to baseline afterwards (Chapter 3). During a longitudinal 7-year follow-up, median hsCRP-levels did not change (Chapter 6). In this study no associations existed between hsCRP and the development of clinical morbidity, although in our cross-sectional analysis on prevalence of the metabolic syndrome patients with this syndrome had higher hsCRP-levels (Chapter 7). Overall, although a recent published paper showed promising findings on the role of hsCRP-levels as early predictive marker for treatment-related CVD,[33] findings from the current studies do not provide supportive data on the use of hsCRP as marker for subclinical vascular damage.

Biomarker for vascular structure: carotid IMT

Median IMT does not change up to one year after BEP-chemotherapy (Chapter 3), however, a subgroup of patients had increases in their IMT (41%) as well as larger increases in CECs during chemotherapy. Additionally, IMT was higher in testicular cancer survivors with the metabolic syndrome, a subgroup at increased risk for CVD (Chapter 7). In our follow-up study in 7-year testicular cancer survivors the median IMT increased during follow-up, but was not different from healthy subjects (Chapter 6), in line with previous reports.[20,21] Interestingly, testicular cancer survivors had a higher age-related increase in IMT. This may point to accelerated atherosclerosis. Moreover, 7-year survivors with Raynaud's phenomenon secondary to chemotherapy had a higher IMT and a steeper IMT-increase than patients without this phenomenon. Because of the proposed common cause for both increases in IMT and the development of Raynaud's phenomenon, i.e. chemotherapy-related endothelial damage, this important observation deserves further study. Possibly, development of this phenomenon after chemotherapy may be a convenient clinical biomarker for patients with a higher risk for treatment-related CVD. Taking data from our different studies together we conclude that carotid IMT is a marker that is affected in a subgroup of patients with augmented vascular damage, whereas short-term changes in IMT are not distinctive as a single subclinical biomarker for vascular damage.

Biomarkers for cardiac status

Long-term testicular cancer survivors have a higher rate of diastolic cardiac dysfunction.[13,36] So far no clinical studies have investigated whether BEP-chemotherapy induces early changes in cardiac status, whereas early signs for endothelial damage have been reported. Although we observed a decline in diastolic cardiac parameters within 10 months after BEP-chemotherapy (Chapter 4), early changes in cardiac biomarkers and echocardiography parameters were not predictive for late systolic or diastolic dysfunction (Chapter 5). Cardiac abnormalities were mild in 7-year survivors, however, prolonged follow-up is needed to explore whether these subclinical diastolic abnormalities will further progress to clinical cardiac dysfunction.

3. Temporary phenomena for cardiovascular damage during BEP-chemotherapy

Vascular damage: release of CECs

CECs are shed from the vessel wall upon endothelial injury, and increased numbers in the blood can be regarded a temporary sign of chemotherapy-related vascular damage. Levels of CECs increase during and after completion of BEP-chemotherapy (Chapter 3), with larger changes in patients with increases in their carotid IMT and with acute treatment-related cardiovascular events. Moreover, cumulative changes in CECs correlate with other vascular damage biomarkers, such as vWF and sICAM-1. Therefore, temporary increases in CEC-levels may well be useful as marker for early treatment-related vascular damage.

Cardiac damage: troponin

Increases in serum levels of troponin, released from damaged cardiomyocytes, are a temporary sign for cardiac injury.[71] Increases in troponin I occurred in 23% of patients during BEP-chemotherapy, however, these increases were not associated with changes in echocardiography parameters or clinical cardiac morbidity during follow-up (Chapter 5). This may relate to presence of mainly mild cardiac abnormalities at follow-up, as well as the additional contribution of cardiovascular risk factors to the declines in diastolic cardiac function parameters.

4. Prevention of cardiovascular damage during and after completion of BEP-chemotherapy

So far, no intervention studies have investigated strategies to prevent cardiovascular damage during BEP-chemotherapy. Two approaches can be feasible, i.e. concomitant treatment with a vasculo-protective drug or treatment with less toxic regimens.

The relation between carotid IMT-progression and increases in serum lipids in testicular cancer survivors (Chapter 6) provides a rationale for intervention with a statin to decrease the development of accelerated atherosclerosis. Statins have lipid-lowering as well as anti-inflammatory properties, thereby exerting endothelial protection via pleiotropic effects.[197] Additional candidates for prevention of chemotherapy-related endothelial damage can be drugs with proven effects on decreasing the progression of atherosclerosis in other patient populations, e.g. oral antidiabetic agents like metformin.[198]

Alternatively, treatment with less toxic regimens can lower the risk for vascular complications. In this respect, an alternative regimen consists of 4 cycles of etoposide-cisplatin (EP) combination chemotherapy in IGCCC good prognosis testicular cancer patients, instead of 3 BEP-courses. Both regimens are comparable in terms of anti-tumour efficacy.[199] However, the higher cumulative cisplatin-dose in the 4xEP-regimen is offset by the absence of bleomycin, which may relevantly contribute to the amount of endothelial damage. In a large cohort of testicular cancer survivors, the cumulative bleomycin-dose was the only significant predictor for development of Raynaud's phenomenon.[151] In addition, one study reported that in a group of 82 testicular cancer patients treated with the 4xEP-regimen, none developed Raynaud's phenomenon.[200] The finding from our longitudinal follow-up study that development of this symptom is associated with a higher carotid IMT also indicates that omission of bleomycin from the treatment regimens causes less vascular damage. Besides systemic endothelial

damage, the 4xEP-regimen prohibits development of pulmonary toxicity, a sometimes fatal complication.[11]

Prior to initiation of such intervention trails, a suitable endpoint for endothelial damage has to be defined. Based on the findings in our prospective study in a comprehensive panel of biomarkers (Chapter 3), patients with early increases in CECs, persisting high vWF-levels and an IMT-increase after BEP-chemotherapy appear to represent a subgroup with augmented vascular damage. In this respect, a combination of these markers may serve as a biomarker panel to nail-down the vascular damage phenotype and serve as an intermediate endpoint for treatment-related endothelial damage.

5. Follow-up after completion of treatment

Most chemotherapy-treated patients will remain under oncological follow-up for 10 years after treatment. In addition to detection of a possible tumour recurrence, recognition of potential late treatment-related effects can be pursued. Up to now no data is scarce on the design of an effective follow-up schedule. For childhood cancer survivors a detailed consensus-based guideline has been developed providing individualised recommendations on the contents and frequency of follow-up visits.[45] These recommendations can serve as a framework for adult cancer survivor follow-up.

Monitoring for and treatment of cardiovascular risk factors during follow-up is crucial to lower the chance of progression of overt CVD. In our studies we observed associations between the development of cardiovascular risk factors, a higher IMT-progression rate (Chapter 6) and larger declines in diastolic cardiac function (Chapter 5), underscoring the relevance of these risk factors in the risk for late CVD. In the absence of evidence-based recommendations, a pragmatic approach can be pursued by estimation of cardiovascular risk factors at routine visits to the outpatient clinic. This can be done at regular intervals, e.g. every two years, starting at one year after start of BEP-chemotherapy. At these visits presence of risk factors for CVD, such as traditional risk factors and risk behaviour, can be determined and treated according to guidelines for the general population. Because of the signs of accelerated atherosclerosis in testicular cancer survivors (Chapter 6 & 7), even more stringent thresholds for cardiovascular risk factors may be indicated, such as used in secondary prevention. Through a joint collaboration between oncologists, general practitioners, internists, vascular specialists and cardiologists, optimal care for these survivors can be pursued. This collaboration can be based on a shared care model as is currently investigated and implemented in childhood cancer survivors.[201]

Testicular cancer as a model for healthy cancer survivorship

In addition to pursuing healthy survivorship for testicular cancer patients, findings from these studies may be translated to other young adult cancer populations with a favourable cancer-related prognosis, such as adjuvantly treated breast, cervical or colorectal cancer patients.

So far, childhood cancer survivors studies have generated most data on late complications after cancer treatmens.[45,202] However, these findings can not be directly translated to the (young) adult cancer patient population, as treatment regimens for childhood cancer largely differ from regimens used in adult cancer patients. Moreover, specific risks in childhood cancer

survivors relate to the immaturity of the organ systems. Testicular cancer patients are a young and fairly homogeneous population with little additional co-morbidities. These patients are treated with uniform treatment regimens and remain under active surveillance for several years after completion of treatment, usually in referral hospitals experienced in treatment of this patient population. By exploring mechanisms and biomarkers for treatment-related complications in testicular cancer patients, this population is a model to pave the way for effective evidence-based strategies to decrease the development of treatment-related complications in young adult cancer survivors.

Summary in Dutch

Chapter 10

ACHTERGROND

Zaadbal (testis) kanker is de meest voorkomende vorm van kanker bij mannen van 20-35 jaar oud. Door de ontwikkeling van platinabevattende chemotherapieschema's eind jaren zeventig[4] is uitgezaaide testiskanker een goed te behandelen vorm van kanker geworden, met een 10-jaars overleving van ongeveer 80%.[2,5] Als gevolg van deze gunstige prognose en een stijgende incidentie[1,5] wordt de groep testiskankeroverlevers steeds groter. Omdat zij in principe een normale levensverwachting hebben wordt het risico op nadelige gevolgen van de behandeling bij deze overlevers in toenemende mate van belang geacht.[2,6] Belangrijke voorbeelden van deze nadelige gevolgen zijn verminderde vruchtbaarheid, schade aan zenuwtakjes en een verhoogd risico op het ontwikkelen van tweede tumoren en hart- en vaatziekten.[6,7]
Bij testiskankerpatiënten kunnen tijdens of kort na behandeling met chemotherapie cardiovasculaire problemen ontstaan, zoals arteriële of veneuze thrombo-embolische complicaties.[8,9] Daarnaast zijn het optreden van het fenomeen van Raynaud[10] en (soms fataal verlopende) longschade[11] voorbeelden van uitingen van vasculaire schade door deze curatieve behandeling. Naast vroege cardiovasculaire complicaties hebben testiskankeroverlevers een verhoogd risico op hart- en vaatziekten na voltooiing van de chemotherapiebehandeling.[16,193] Verschillende studies hebben bovendien aangetoond dat testiskankeroverlevers meer subklinische atherosclerose hebben dan gezonde mannen van dezelfde leeftijd.[18-21]

Uit zowel patiënten- als laboratoriumonderzoek is duidelijk geworden dat schade aan de bekleding van bloedvaten (endotheel) door chemotherapie een belangrijk mechanisme is voor het ontstaan van vasculaire complicaties. Naast directe endotheelschade kunnen cardiovasculaire risicofactoren (zoals overgewicht, hypertensie, dyslipidemie en insulineresistentie) en hormonale veranderingen bijdragen aan het optreden van hart- en vaatziekten bij testiskankeroverlevers.[31]

Het ontstaan van hart- en vaatziekten is een geleidelijk proces. Endotheelschade kan aanleiding geven tot subklinische veranderingen aan de vaatwand, die zich in de loop van verscheidene jaren verder kan ontwikkelen tot klinisch relevante atherosclerose. Vroegtijdige herkenning van patiënten met een verhoogd risico op het ontwikkelen van atherosclerose is belangrijk, omdat hiermee het ontstaan van deze behandelingsgerelateerde complicaties kan worden vertraagd of tot staan gebracht.

Gevoelige voorspellers (biomarkers) voor het optreden van behandelingsgerelateerde endotheelschade zouden kunnen worden gebruikt om per patiënt het risico op het ontstaan van cardiovasculaire complicaties in te schatten. Als zulke biomarkers beschikbaar zijn kunnen de behandeling en follow-up van testiskankerpatiënten worden geoptimaliseerd en geïndividualiseerd. Ook kunnen specifieke biomarkers inzicht geven in de wijze waarop chemotherapiegerelateerde endotheelschade ontstaat, en daarmee aanknopingspunten bieden voor het voorkomen van deze complicaties. Uiteindelijk zouden zulke biomarkers ook kunnen dienen als maat voor de uitgebreidheid van de endotheelschade die ontstaat door kankerbehandelingen. Hiermee kunnen interventiestudies kunnen worden uitgevoerd om de mate van cardiovasculaire schade door chemotherapie te verminderen of te voorkomen.

DOEL VAN HET PROEFSCHRIFT

Dit proefschrift is gericht op het vinden van gevoelige biomarkers voor subklinische behandelingsgerelateerde cardiovasculaire schade bij testiskankerpatiënten behandeld met cisplatinebevattende chemotherapie.

SAMENVATTING

Het bepalen van de linker ventrikel ejectiefractie is de meest gebruikte manier om cardiovasculaire schade door kankerbehandelingen te meten. Het nadeel van de ejectiefractie is dat deze methode de toegebrachte hartschade kan onderschatten, omdat subklinische schade aan het hart niet direct hoeft te leiden tot een verminderde pompfunctie. In **hoofdstuk 2** hebben we een overzicht gegeven van de voor- en nadelen van verschillende methoden, waaronder de ejectiefractie, waarmee cardiovasculaire schade tijdens en na kankerbehandelingen kan worden gemeten. Ook zoeken we naar bewijs in de literatuur voor de toepasbaarheid van de verschillende meetmethoden. Onze literatuurstudie laat zien dat de op dit moment gebruikte methoden om cardiovasculaire schade te meten slechts weinig harde wetenschappelijke onderbouwing hebben. Op basis van deze bevindingen benadrukken we het belang van het doen van onderzoek naar betrouwbare en gevoelige methoden om cardiovasculaire schade door kankerbehandelingen te meten.

Veranderingen in biomarkers voor subklinische vaatschade kunnen worden gebruikt om patiënten aan te wijzen die een verhoogd risico op chemotherapiegerelateerde complicaties hebben. In een studie bij testiskankerpatiënten hebben we veranderingen in een panel van verschillende vasculaire biomarkers gemeten. Dit panel bestond uit circulerende endotheelcellen (CECs), plasma biomarkers voor endotheelactivatie, inflammatie en verhoogde stollingsneiging en de vaatstructuur (Intima Media Dikte van de arteria carotis [IMT]; **hoofdstuk 3**). In deze studie bleken de patiënten waarin de CECs, markers voor endotheelactivatie en de IMT toenamen te behoren tot een subgroep met een hogere mate van chemotherapiegerelateerde vaatschade. Door deze groep langer te vervolgen zal duidelijk worden of veranderingen in CECs en de biomarkers voor endotheelactivatie het ontstaan van late cardiovasculaire problemen bij testiskankeroverlevers kunnen voorspellen.

Lange termijn testiskankeroverlevers hebben een verhoogde kans om diastolische dysfunctie van het hart te ontwikkelen.[13,36] We hebben bij testiskankerpatiënten vroege veranderingen in hartfunctie gemeten met echocardiografie, en onderzocht of deze veranderingen mogelijk een voorbode zijn voor latere cardiale dysfunctie. Binnen een jaar na start van chemotherapie vonden we een verslechtering van de hartfunctie, gemeten met gevoelige diastolische parameters en de biomarker N-terminal pro Brain natriuretisch peptide (NT-proBNP) in het bloed (**hoofdstuk 4**). Vervolgens hebben we bij deze patiënten mediaan 7 jaar na start van de behandeling opnieuw een echocardiogram gemaakt. In deze periode bleek de incidentie van een abnormale diastolische functie toegenomen te zijn (**hoofdstuk 5**). Het aantal patiënten met een abnormale pompfunctie bleef laag aangezien slechts één van de 37 patiënten (2,7%) een te lage linker ventrikel ejectiefractie had. Stijgingen van cardiale biomarkers in het bloed bleken niet voorspellend te zijn voor het later optreden van een abnormale hartfunctie. Wel vonden we in beide studies dat een verslechtering van de diastolische hartfunctie het meest

uitgesproken was bij patiënten die hoge bloeddruk en overgewicht hadden ontwikkeld. Deze bevindingen benadrukken het belang van aandacht voor het opsporing en behandeling van cardiovasculaire risicofactoren bij testiskankeroverlevers.

De IMT van de arteria carotis is een biomarker voor atherosclerose: een dikkere IMT geldt als onafhankelijke voorspeller voor het optreden van cardiovasculaire events in de algemene populatie.[143] Een versnelde toename van de IMT is een teken van atherosclerose, en is geassocieerd met een hoger risico op hart- en vaatziekten. We hebben bij een groep testiskankerpatiënten de IMT vóór, 11 maanden en 7 jaar na chemotherapiebehandeling gemeten. Tijdens deze longitudinale follow-up nam de IMT toe (**hoofdstuk 6**). Vergeleken met een groep gezonde mannen van dezelfde leeftijd was de mediane IMT bij de 7-jaars meting niet verschillend, maar wel bleek de leeftijdsgerelateerde toename van de IMT groter bij de testiskankeroverlevers. De mediane jaarlijkse IMT toename na behandeling was gerelateerd aan veranderingen in serum lipiden. Het bleek dat patiënten die het fenomeen van Raynaud hadden ontwikkeld na de chemotherapiebehandeling een hogere IMT hadden tijdens follow-up, en een hogere leeftijdsgerelateerde toename van de IMT. Deze relatie zou kunnen betekenen dat het fenomeen van Raynaud een klinische biomarker voor atherosclerose bij testiskankeroverlevers kan zijn.

Testiskankeroverlevers hebben een hogere prevalentie van het metabool syndroom dan gezonde mannelijke leeftijdsgenoten. Het metabool syndroom is een cluster van cardiovasculaire risicofactoren, en is geassocieerd met een toegenomen risico op het ontstaan van hart- en vaatziekten. In **hoofdstuk 7** wordt beschreven dat in een groep testiskankeroverlevers die tenminste 3 jaar na de chemotherapiebehandeling zijn, het metabool syndroom aanwezig is bij 25%. Dat is twee keer hoger dan in een groep gezonde mannen van dezelfde leeftijd. Afzonderlijke componenten van het metabool syndroom traden in deze patiëntengroep relatief snel na de chemotherapiebehandeling al op: overgewicht na mediaan 1.7, hypercholesterolemie na mediaan 0.9 en hypertensie na 5.1 jaar. Patiënten met het metabool syndroom hadden een dikkere IMT en hogere inflammatie- en stollingsparameters in het bloed. Het snelle optreden en het verhoogde voorkomen van het metabool syndroom bij testiskankeroverlevers benadrukt opnieuw het belang van vroegtijdige opsporing en behandeling van deze risicofactoren. Het ontwikkelen van richtlijnen waarin systematisch aandacht wordt besteed aan het optreden van deze risicofactoren lijkt van belang, om de kans op hart- en vaatziekten bij testiskankeroverlevers te verminderen.

Om inzicht te krijgen in moleculaire processen die een rol spelen bij het ontstaan van chemotherapiegerelateerde schade hebben we een cDNA microarray analyse uitgevoerd bij endotheelcellen die waren blootgesteld aan de cytostatica bleomycine en cisplatine. In deze analyse vonden we significante veranderingen in genexpressie van vele genen, waarbij de genen *GDF15*, *ATF3* en *AREG* uit verschillende experimenten naar voren kwamen. Daarnaast vonden we veranderingen in cellulaire signaalroutes die betrokken zijn bij celdood en inflammatie (**hoofdstuk 8**). Vervolgens bestudeerde we daarom de eiwitconcentratie van GDF15. Dit cytokine bleek, net als het RNA *in vitro*, toe te nemen in het plasma van testiskankerpatiënten tijdens de behandeling met bleomycine- en cisplatine. Deze studie laat zien dat cDNA microarray analyse een techniek is die richting kan geven om op een zinvolle

'translationele' manier te zoeken naar nieuwe biomarkers voor chemotherapiegerelateerde vaatschade. Ook kan deze methode meer inzicht bieden in de cellulaire processen die een rol spelen bij het ontstaan van endotheelschade door chemotherapie. Aanvullend onderzoek in het laboratorium en bij patiënten is gewenst om deze bevindingen verder te analyseren.

In **hoofdstuk 9** vatten we de bevindingen uit de voorgaande hoofdstukken samen. Daarna schetsen we een model waarin verschillende manieren waarop cardiovasculaire schade tijdens en na kankerbehandelingen worden weergegeven. Aan de hand van dit model worden de bevindingen uit onze studies en uit de literatuur samengevat.
Met de studies zoals beschreven in dit proefschrift zijn we meer te weten gekomen over mechanismen die bijdragen aan het ontstaan van cardiovasculaire complicaties bij testiskankerpatiënten. Daarnaast hebben we verschillende biomarkers gevonden die mogelijk een rol kunnen spelen bij het eerder opsporen van chemotherapiegerelateerde cardiovasculaire schade. Patiënten met testiskanker zijn over het algemeen een jonge en relatief homogene populatie, die meestal weinig andere comorbiditeit heeft. Deze mannen worden met standaard chemotherapieschema's behandeld, en blijven vaak nog tot jaren na hun behandeling onder controle bij een medisch oncoloog. Onderzoek in deze populatie kan model staan voor onderzoek naar lange termijn effecten in andere groepen kankeroverlevers. Uiteindelijk kunnen bevindingen bij deze patiënten als basis dienen voor het ontwikkelen van 'evidence-based' strategieën om de kans op behandelingsgerelateerde complicaties bij jongvolwassen kankeroverlevers te verminderen.

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Bibliography

1. IKCnet. http://www.ikcnet.nl/page.php?nav_id=41&id=2748; 2009
2. Feldman DR, Bosl GJ, Sheinfeld J et al: Medical treatment of advanced testicular cancer. JAMA 299:672-684, 2008
3. International Germ Cell Consensus Classification: a prognostic factor-based staging system for metastatic germ cell cancers. International Germ Cell Cancer Collaborative Group: J Clin Oncol 15:594-603, 1997
4. Einhorn LH, Donohue J: Cis-diamminedichloroplatinum, vinblastine, and bleomycin combination chemotherapy in disseminated testicular cancer. Ann Intern Med 87:293-298, 1977
5. Verdecchia A, Francisci S, Brenner H et al: Recent cancer survival in Europe: a 2000-02 period analysis of EUROCARE-4 data. Lancet Oncol 8:784-796, 2007
6. Travis LB, Beard C, Allan JM et al: Testicular cancer survivorship: research strategies and recommendations. J Natl Cancer Inst 102:1114-1130, 2010
7. van den Belt-Dusebout AW, de Wit R, Gietema JA et al: Treatment-specific risks of second malignancies and cardiovascular disease in 5-year survivors of testicular cancer. J Clin Oncol 25:4370-4378, 2007
8. Nuver J, Smit AJ, van der Meer J et al: Acute chemotherapy-induced cardiovascular changes in patients with testicular cancer. J Clin Oncol 23:9130-9137, 2005
9. Weijl NI, Rutten MF, Zwinderman AH et al: Thromboembolic events during chemotherapy for germ cell cancer: a cohort study and review of the literature. J Clin Oncol 18:2169-2178, 2000
10. Vogelzang NJ, Bosl GJ, Johnson K et al: Raynaud's phenomenon: a common toxicity after combination chemotherapy for testicular cancer. Ann Intern Med 95:288-292, 1981
11. Sleijfer S: Bleomycin-induced pneumonitis. Chest 120:617-624, 2001
12. Huddart RA, Norman A, Shahidi M et al: Cardiovascular disease as a long-term complication of treatment for testicular cancer. J Clin Oncol 21:1513-1523, 2003
13. Meinardi MT, Gietema JA, van der Graaf WT et al: Cardiovascular morbidity in long-term survivors of metastatic testicular cancer. J Clin Oncol 18:1725-1732, 2000
14. Strumberg D, Brugge S, Korn MW et al: Evaluation of long-term toxicity in patients after cisplatin-based chemotherapy for non-seminomatous testicular cancer. Ann Oncol 13:229-236, 2002
15. van den Belt-Dusebout AW, Nuver J, de Wit R et al: Long-term risk of cardiovascular disease in 5-year survivors of testicular cancer. J Clin Oncol 24:467-475, 2006
16. Haugnes HS, Wethal T, Aass N et al: Cardiovascular risk factors and morbidity in long-term survivors of testicular cancer: a 20-year follow-up study. J Clin Oncol, 2010
17. Fossa SD, Aass N, Harvei S et al: Increased mortality rates in young and middle-aged patients with malignant germ cell tumours. Br J Cancer 90:607-612, 2004
18. Haugnes HS, Aass N, Fossa SD et al: Components of the metabolic syndrome in long-term survivors of testicular cancer. Ann Oncol 18:241-248, 2007
19. Nuver J, Smit AJ, Wolffenbuttel BH et al: The metabolic syndrome and disturbances in hormone levels in long-term survivors of disseminated testicular cancer. J Clin Oncol 23:3718-3725, 2005
20. Vaughn DJ, Palmer SC, Carver JR et al: Cardiovascular risk in long-term survivors of testicular cancer. Cancer 112:1949-1953, 2008
21. Nuver J, Smit AJ, Sleijfer DT et al: Microalbuminuria, decreased fibrinolysis, and inflammation as early signs of atherosclerosis in long-term survivors of disseminated testicular cancer. Eur J Cancer 40:701-706, 2004
22. Lechner D, Kollars M, Gleiss A et al: Chemotherapy-induced thrombin generation via procoagulant endothelial microparticles is independent of tissue factor activity. J Thromb Haemost 5:2445-2452, 2007
23. L'Azou B, Fernandez P, Bareille R et al: In vitro endothelial cell susceptibility to xenobiotics: comparison of three cell types. Cell Biol Toxicol 21:127-137, 2005
24. Nuver J, De Haas EC, Van Zweeden M et al: Vascular damage in testicular cancer patients: A study on endothelial activation by bleomycin and cisplatin in vitro. Oncol Rep 23:247-253, 2010
25. Yu M, Han J, Cui P et al: Cisplatin up-regulates ICAM-1 expression in endothelial cell via a NF-kappaB dependent pathway. Cancer Sci 99:391-397, 2008
26. Ishii H, Takada K: Bleomycin induces E-selectin expression in cultured umbilical vein endothelial cells by increasing its mRNA levels through activation of NF-kappaB/Rel. Toxicol Appl Pharmacol 184:88-97, 2002
27. Shi Y, Inoue S, Shinozaki R et al: Release of cytokines from human umbilical vein endothelial cells treated with platinum compounds in vitro. Jpn J Cancer Res 89:757-767, 1998

28. Gietema JA, Meinardi MT, Messerschmidt J et al: Circulating plasma platinum more than 10 years after cisplatin treatment for testicular cancer. Lancet 355:1075-1076, 2000
29. Hansson GK, Robertson AK, Soderberg-Naucler C: Inflammation and atherosclerosis. Annu Rev Pathol 1:297-329, 2006
30. Deanfield JE, Halcox JP, Rabelink TJ: Endothelial function and dysfunction: testing and clinical relevance. Circulation 115:1285-1295, 2007
31. de Haas EC, Oosting SF, Lefrandt JD et al: The metabolic syndrome in cancer survivors. Lancet Oncol 11:193-203, 2010
32. Ma RC, Tong PC: Testosterone levels and cardiovascular disease. Heart 96:1787-1788, 2010
33. Vasan RS: Biomarkers of cardiovascular disease: molecular basis and practical considerations. Circulation 113:2335-2362, 2006
34. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework: Clin Pharmacol Ther 69:89-95, 2001
35. Philips B, Ball C, Sackett D, Badenoch D, Straus S, Haynes B et al. Oxford Centre for Evidence-based Medicine Levels of Evidence . http://www.cebm.net/index.aspx?o=1025 2001.
36. Nuver J, Smit AJ, Sleijfer DT et al: Left ventricular and cardiac autonomic function in survivors of testicular cancer. Eur J Clin Invest 35:99-103, 2005
37. Ferlay J, Autier P, Boniol M et al: Estimates of the cancer incidence and mortality in Europe in 2006. Ann Oncol 18:581-592, 2007
38. Pulte D, Gondos A, Brenner H: Trends in 5- and 10-year survival after diagnosis with childhood hematologic malignancies in the United States, 1990-2004. J Natl Cancer Inst 100:1301-1309, 2008
39. Trudeau M, Charbonneau F, Gelmon K et al: Selection of adjuvant chemotherapy for treatment of node-positive breast cancer. Lancet Oncol 6:886-898, 2005
40. Clarke M, Collins R, Darby S et al: Effects of radiotherapy and of differences in the extent of surgery for early breast cancer on local recurrence and 15-year survival: an overview of the randomised trials. Lancet 366:2087-2106, 2005
41. Meinardi MT, van der Graaf WT, van Veldhuisen DJ et al: Detection of anthracycline-induced cardiotoxicity. Cancer Treat Rev 25:237-247, 1999
42. Carver JR, Shapiro CL, Ng A et al: American Society of Clinical Oncology clinical evidence review on the ongoing care of adult cancer survivors: cardiac and pulmonary late effects. J Clin Oncol 25:3991-4008, 2007
43. Mitani I, Jain D, Joska TM et al: Doxorubicin cardiotoxicity: prevention of congestive heart failure with serial cardiac function monitoring with equilibrium radionuclide angiocardiography in the current era. J Nucl Cardiol 10:132-139, 2003
44. Hunt SA, Abraham WT, Chin MH et al: ACC/AHA 2005 Guideline Update for the Diagnosis and Management of Chronic Heart Failure in the Adult: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Writing Committee to Update the 2001 Guidelines for the Evaluation and Management of Heart Failure): developed in collaboration with the American College of Chest Physicians and the International Society for Heart and Lung Transplantation: endorsed by the Heart Rhythm Society. Circulation 112:e154-e235, 2005
45. Children's Oncology Group., Hudson MM, Landier W, Bhatia S. Long-term follow-up guidelines for survivors of childhood, adolescent, and young adult cancers. Sections 6-37: chemotherapy. http://www.guideline.gov/summary/summary.aspx?doc_id=10770&nbr=005598; 2006
46. Schimmel KJ, Richel DJ, van den Brink RB et al: Cardiotoxicity of cytotoxic drugs. Cancer Treat Rev 30:181-191, 2004
47. Gaya AM, Ashford RF: Cardiac complications of radiation therapy. Clin Oncol (R Coll Radiol) 17:153-159, 2005
48. Suter TM, Procter M, van Veldhuisen DJ et al: Trastuzumab-associated cardiac adverse effects in the herceptin adjuvant trial. J Clin Oncol 25:3859-3865, 2007
49. Zhou S, Starkov A, Froberg MK et al: Cumulative and irreversible cardiac mitochondrial dysfunction induced by doxorubicin. Cancer Res 61:771-777, 2001
50. Jannazzo A, Hoffman J, Lutz M: Monitoring of anthracycline-induced cardiotoxicity. Ann Pharmacother 42:99-104, 2008
51. Ewer MS, Lenihan DJ: Left ventricular ejection fraction and cardiotoxicity: is our ear really to the ground? J Clin Oncol 26:1201-1203, 2008

52. Lester SJ, Tajik AJ, Nishimura RA et al: Unlocking the mysteries of diastolic function: deciphering the Rosetta Stone 10 years later. J Am Coll Cardiol 51:679-689, 2008
53. Jurcut R, Wildiers H, Ganame J et al: Detection and monitoring of cardiotoxicity-what does modern cardiology offer? Support Care Cancer 16:437-445, 2008
54. Swain SM, Whaley FS, Ewer MS: Congestive heart failure in patients treated with doxorubicin: a retrospective analysis of three trials. Cancer 97:2869-2879, 2003
55. Jensen BV, Skovsgaard T, Nielsen SL: Functional monitoring of anthracycline cardiotoxicity: a prospective, blinded, long-term observational study of outcome in 120 patients. Ann Oncol 13:699-709, 2002
56. Nousiainen T, Jantunen E, Vanninen E et al: Early decline in left ventricular ejection fraction predicts doxorubicin cardiotoxicity in lymphoma patients. Br J Cancer 86:1697-1700, 2002
57. Sohn DW, Chai IH, Lee DJ et al: Assessment of mitral annulus velocity by Doppler tissue imaging in the evaluation of left ventricular diastolic function. J Am Coll Cardiol 30:474-480, 1997
58. Wang J, Khoury DS, Thohan V et al: Global diastolic strain rate for the assessment of left ventricular relaxation and filling pressures. Circulation 115:1376-1383, 2007
59. Tassan-Mangina S, Codorean D, Metivier M et al: Tissue Doppler imaging and conventional echocardiography after anthracycline treatment in adults: early and late alterations of left ventricular function during a prospective study. Eur J Echocardiogr 7:141-146, 2006
60. Tjeerdsma G, Meinardi MT, van der Graaf WT et al: Early detection of anthracycline induced cardiotoxicity in asymptomatic patients with normal left ventricular systolic function: autonomic versus echocardiographic variables. Heart 81:419-423, 1999
61. Marchandise B, Schroeder E, Bosly A et al: Early detection of doxorubicin cardiotoxicity: interest of Doppler echocardiographic analysis of left ventricular filling dynamics. Am Heart J 118:92-98, 1989
62. Morandi P, Ruffini PA, Benvenuto GM et al: Serum cardiac troponin I levels and ECG/Echo monitoring in breast cancer patients undergoing high-dose (7 g/m(2)) cyclophosphamide. Bone Marrow Transplant 28:277-282, 2001
63. Mercuro G, Cadeddu C, Piras A et al: Early epirubicin-induced myocardial dysfunction revealed by serial tissue Doppler echocardiography: correlation with inflammatory and oxidative stress markers. Oncologist 12:1124-1133, 2007
64. Kapusta L, Thijssen JM, Groot-Loonen J et al: Discriminative ability of conventional echocardiography and tissue Doppler imaging techniques for the detection of subclinical cardiotoxic effects of treatment with anthracyclines. Ultrasound Med Biol 27:1605-1614, 2001
65. Nagy AC, Tolnay E, Nagykalnai T et al: Cardiotoxicity of anthracycline in young breast cancer female patients: the possibility of detection of early cardiotoxicity by TDI. Neoplasma 53:511-517, 2006
66. Lotrionte M, Palazzoni G, Natali R et al: Appraising cardiotoxicity associated with liposomal doxorubicin by means of tissue Doppler echocardiography end-points Rationale and design of the LITE (Liposomal doxorubicin-Investigational chemotherapy-Tissue Doppler imaging Evaluation) randomized pilot study. Int J Cardiol, 2008
67. Broeyer FJ, Osanto S, Ritsema van Eck HJ et al: Evaluation of biomarkers for cardiotoxicity of anthracyclin-based chemotherapy. J Cancer Res Clin Oncol, 2008
68. Nakamae H, Tsumura K, Hino M et al: QT dispersion as a predictor of acute heart failure after high-dose cyclophosphamide. Lancet 355:805-806, 2000
69. Nakamae H, Tsumura K, Akahori M et al: QT dispersion correlates with systolic rather than diastolic parameters in patients receiving anthracycline treatment. Intern Med 43:379-387, 2004
70. Braunwald E: Biomarkers in heart failure. N Engl J Med 358:2148-2159, 2008
71. Steen H, Giannitsis E, Futterer S et al: Cardiac troponin T at 96 hours after acute myocardial infarction correlates with infarct size and cardiac function. J Am Coll Cardiol 48:2192-2194, 2006
72. Okumura H, Iuchi K, Yoshida T et al: Brain natriuretic peptide is a predictor of anthracycline-induced cardiotoxicity. Acta Haematol 104:158-163, 2000
73. Suzuki T, Hayashi D, Yamazaki T et al: Elevated B-type natriuretic peptide levels after anthracycline administration. Am Heart J 136:362-363, 1998
74. Meinardi MT, van Veldhuisen DJ, Gietema JA et al: Prospective evaluation of early cardiac damage induced by epirubicin-containing adjuvant chemotherapy and locoregional radiotherapy in breast cancer patients. J Clin Oncol 19:2746-2753, 2001

75. Sandri MT, Salvatici M, Cardinale D et al: N-terminal pro-B-type natriuretic peptide after high-dose chemotherapy: a marker predictive of cardiac dysfunction? Clin Chem 51:1405-1410, 2005
76. Dodos F, Halbsguth T, Erdmann E et al: Usefulness of myocardial performance index and biochemical markers for early detection of anthracycline-induced cardiotoxicity in adults. Clin Res Cardiol, 2008
77. Cardinale D, Sandri MT, Colombo A et al: Prognostic value of troponin I in cardiac risk stratification of cancer patients undergoing high-dose chemotherapy. Circulation 109:2749-2754, 2004
78. de Vos FY, Willemse PH, de Vries EG et al: Endothelial cell effects of cytotoxics: balance between desired and unwanted effects. Cancer Treat Rev 30:495-513, 2004
79. Fichtner F, Koslowski R, Augstein A et al: Bleomycin induces IL-8 and ICAM-1 expression in microvascular pulmonary endothelial cells. Exp Toxicol Pathol 55:497-503, 2004
80. Periard D, Boulanger CM, Eyer S et al: Are circulating endothelial-derived and platelet-derived microparticles a pathogenic factor in the cisplatin-induced stroke? Stroke 38:1636-1638, 2007
81. Perik PJ, de Vries EG, Boomsma F et al: The relation between soluble apoptotic proteins and subclinical cardiotoxicity in adjuvant-treated breast cancer patients. Anticancer Res 26:3803-3811, 2006
82. Duquaine D, Hirsch GA, Chakrabarti A et al: Rapid-onset endothelial dysfunction with adriamycin: evidence for a dysfunctional nitric oxide synthase. Vasc Med 8:101-107, 2003
83. Dolan ME, Newbold KG, Nagasubramanian R et al: Heritability and linkage analysis of sensitivity to cisplatin-induced cytotoxicity. Cancer Res 64:4353-4356, 2004
84. Duan S, Bleibel WK, Huang RS et al: Mapping genes that contribute to daunorubicin-induced cytotoxicity. Cancer Res 67:5425-5433, 2007
85. Marsh S, Paul J, King CR et al: Pharmacogenetic assessment of toxicity and outcome after platinum plus taxane chemotherapy in ovarian cancer: the Scottish Randomised Trial in Ovarian Cancer. J Clin Oncol 25:4528-4535, 2007
86. Wojnowski L, Kulle B, Schirmer M et al: NAD(P)H oxidase and multidrug resistance protein genetic polymorphisms are associated with doxorubicin-induced cardiotoxicity. Circulation 112:3754-3762, 2005
87. Beauclair S, Formento P, Fischel JL et al: Role of the HER2 [Ile655Val] genetic polymorphism in tumorogenesis and in the risk of trastuzumab-related cardiotoxicity. Ann Oncol 18:1335-1341, 2007
88. Flotats A, Carrio I: Non-invasive in vivo imaging of myocardial apoptosis and necrosis. Eur J Nucl Med Mol Imaging 30:615-630, 2003
89. Bengel FM, Schwaiger M: Assessment of cardiac sympathetic neuronal function using PET imaging. J Nucl Cardiol 11:603-616, 2004
90. Estorch M, Carrio I, Berna L et al: Indium-111-antimyosin scintigraphy after doxorubicin therapy in patients with advanced breast cancer. J Nucl Med 31:1965-1969, 1990
91. Carrio I, Lopez-Pousa A, Estorch M et al: Detection of doxorubicin cardiotoxicity in patients with sarcomas by indium-111-antimyosin monoclonal antibody studies. J Nucl Med 34:1503-1507, 1993
92. Valdes Olmos RA, Carrio I, Hoefnagel CA et al: High sensitivity of radiolabelled antimyosin scintigraphy in assessing anthracycline related early myocyte damage preceding cardiac dysfunction. Nucl Med Commun 23:871-877, 2002
93. Carrio I, Estorch M, Berna L et al: Indium-111-antimyosin and iodine-123-MIBG studies in early assessment of doxorubicin cardiotoxicity. J Nucl Med 36:2044-2049, 1995
94. Nousiainen T, Vanninen E, Jantunen E et al: Anthracycline-induced cardiomyopathy: long-term effects on myocardial cell integrity, cardiac adrenergic innervation and fatty acid uptake. Clin Physiol 21:123-128, 2001
95. Valdes Olmos RA, ten Bokkel Huinink WW, ten Hoeve RF et al: Assessment of anthracycline-related myocardial adrenergic derangement by [123I]metaiodobenzylguanidine scintigraphy. Eur J Cancer 31A:26-31, 1995
96. Bergmann SR: Imaging of myocardial fatty acid metabolism with PET. J Nucl Cardiol 14:S118-S124, 2007
97. de Korte MA, de Vries EG, Lub-de Hooge MN et al: (111)Indium-trastuzumab visualises myocardial human epidermal growth factor receptor 2 expression shortly after anthracycline treatment but not during heart failure: A clue to uncover the mechanisms of trastuzumab-related cardiotoxicity. Eur J Cancer, 2007
98. Perik PJ, Lub-de Hooge MN, Gietema JA et al: Indium-111-labeled trastuzumab scintigraphy in patients with human epidermal growth factor receptor 2-positive metastatic breast cancer. J Clin Oncol 24:2276-2282, 2006
99. Bandettini WP, Arai AE: Advances in Clinical Applications of Cardiovascular Magnetic Resonance Imaging.

Heart, 2008

100. Fallah-Rad N, Lytwyn M, Fang T et al: Delayed contrast enhancement cardiac magnetic resonance imaging in trastuzumab induced cardiomyopathy. J Cardiovasc Magn Reson 10:5, 2008

101. Gietema JA, Meinardi MT, van der Graaf WT et al: Syndrome X in testicular-cancer survivors. Lancet 357:228-229, 2001

102. Stefenelli T, Kuzmits R, Ulrich W et al: Acute vascular toxicity after combination chemotherapy with cisplatin, vinblastine, and bleomycin for testicular cancer. Eur Heart J 9:552-556, 1988

103. van den Belt-Dusebout AW, Nuver J, de WR et al: Long-term risk of cardiovascular disease in 5-year survivors of testicular cancer. J Clin Oncol 24:467-475, 2006

104. Vischer UM: von Willebrand factor, endothelial dysfunction, and cardiovascular disease. J Thromb Haemost 4:1186-1193, 2006

105. Rowand JL, Martin G, Doyle GV et al: Endothelial cells in peripheral blood of healthy subjects and patients with metastatic carcinomas. Cytometry A 71:105-113, 2007

106. Blann AD, Woywodt A, Bertolini F et al: Circulating endothelial cells. Biomarker of vascular disease. Thromb Haemost 93:228-235, 2005

107. Strijbos MH, Rao C, Schmitz PI et al: Correlation between circulating endothelial cell counts and plasma thrombomodulin levels as markers for endothelial damage. Thromb Haemost 100:642-647, 2008

108. Strijbos MH, Kraan J, den Bakker MA et al: Cells meeting our immunophenotypic criteria of endothelial cells are large platelets. Cytometry B Clin Cytom 72:86-93, 2007

109. Smirnov DA, Foulk BW, Doyle GV et al: Global gene expression profiling of circulating endothelial cells in patients with metastatic carcinomas. Cancer Res 66:2918-2922, 2006

110. Strijbos MH, Verhoef C, Gratama JW et al: On the origin of (CD105+) circulating endothelial cells. Thromb Haemost 102:347-351, 2009

111. Strijbos MH, Gratama JW, Schmitz PI et al: Circulating endothelial cells, circulating tumour cells, tissue factor, endothelin-1 and overall survival in prostate cancer patients treated with docetaxel. Eur J Cancer, 2010

112. Roodhart JM, Langenberg MH, Vermaat JS et al: Late release of circulating endothelial cells and endothelial progenitor cells after chemotherapy predicts response and survival in cancer patients. Neoplasia 12:87-94, 2010

113. Kas-Deelen AM, Harmsen MC, De Maar EF et al: Acute rejection before cytomegalovirus infection enhances von Willebrand factor and soluble VCAM-1 in blood. Kidney Int 58:2533-2542, 2000

114. Makin AJ, Blann AD, Chung NA et al: Assessment of endothelial damage in atherosclerotic vascular disease by quantification of circulating endothelial cells. Relationship with von Willebrand factor and tissue factor. Eur Heart J 25:371-376, 2004

115. Hodis HN, Mack WJ, LaBree L et al: The role of carotid arterial intima-media thickness in predicting clinical coronary events. Ann Intern Med 128:262-269, 1998

116. Sharma K, Blaha MJ, Blumenthal RS et al: Clinical and research applications of carotid intima-media thickness. Am J Cardiol 103:1316-1320, 2009

117. Taylor AJ, Villines TC, Stanek EJ et al: Extended-release niacin or ezetimibe and carotid intima-media thickness. N Engl J Med 361:2113-2122, 2009

118. Bots ML, Evans GW, Riley WA et al: Carotid intima-media thickness measurements in intervention studies: design options, progression rates, and sample size considerations: a point of view. Stroke 34:2985-2994, 2003

119. Doust JA, Pietrzak E, Dobson A et al: How well does B-type natriuretic peptide predict death and cardiac events in patients with heart failure: systematic review. BMJ 330:625, 2005

120. Ekstein S, Nir A, Rein AJ et al: N-terminal-proB-type natriuretic peptide as a marker for acute anthracycline cardiotoxicity in children. J Pediatr Hematol Oncol 29:440-444, 2007

121. Bryant J, Picot J, Baxter L et al: Use of cardiac markers to assess the toxic effects of anthracyclines given to children with cancer: a systematic review. Eur J Cancer 43:1959-1966, 2007

122. Cheitlin MD, Armstrong WF, Aurigemma GP et al: ACC/AHA/ASE 2003 Guideline Update for the Clinical Application of Echocardiography: summary article. A report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (ACC/AHA/ASE Committee to Update the 1997 Guidelines for the Clinical Application of Echocardiography). J Am Soc Echocardiogr 16:1091-1110, 2003

123. Nagueh SF, Appleton CP, Gillebert TC et al: Recommendations for the evaluation of left ventricular

diastolic function by echocardiography. Eur J Echocardiogr 10:165-193, 2009

124. Kowalski M, Kukulski T, Jamal F et al: Can natural strain and strain rate quantify regional myocardial deformation? A study in healthy subjects. Ultrasound Med Biol 27:1087-1097, 2001

125. Zile MR, Brutsaert DL: New concepts in diastolic dysfunction and diastolic heart failure: Part I: diagnosis, prognosis, and measurements of diastolic function. Circulation 105:1387-1393, 2002

126. Hurrell DG, Nishimura RA, Ilstrup DM et al: Utility of preload alteration in assessment of left ventricular filling pressure by Doppler echocardiography: a simultaneous catheterization and Doppler echocardiographic study. J Am Coll Cardiol 30:459-467, 1997

127. Nikitin NP, Witte KKA: Application of Tissue Doppler Imaging in Cardiology. Cardiology 101:170-184, 2004

128. Brouwer CA, Gietema JA, van den Berg MP et al: Long-term cardiac follow-up in survivors of a malignant bone tumour. Ann Oncol 17:1586-1591, 2006

129. Galderisi M, Marra F, Esposito R et al: Cancer therapy and cardiotoxicity: the need of serial Doppler echocardiography. Cardiovasc Ultrasound 5:4, 2007

130. Galderisi M: Diastolic dysfunction and diastolic heart failure: diagnostic, prognostic and therapeutic aspects. Cardiovasc Ultrasound 3:9, 2005

131. Wierzbowska-Drabik K, Krzeminska-Pakula M, Chrzanowski L et al: Age-dependency of classic and new parameters of diastolic f unction. Echocardiography 25:149-155, 2008

132. Altena R, de Haas EC, Nuver J et al: Evaluation of sub-acute changes in cardiac function after cisplatin-based combination chemotherapy for testicular cancer. Br J Cancer 100:1861-1866, 2009

133. Schillaci G, Pasqualini L, Verdecchia P et al: Prognostic significance of left ventricular diastolic dysfunction in essential hypertension. J Am Coll Cardiol 39:2005-2011, 2002

134. de Boer RA, Voors AA, Muntendam P et al: Galectin-3: a novel mediator of heart failure development and progression. Eur J Heart Fail 11:811-817, 2009

135. Christenson RH, Duh SH, Wu AH et al: Multi-center determination of galectin-3 assay performance characteristics: Anatomy of a novel assay for use in heart failure. Clin Biochem 43:683-690, 2010

136. Kuczmarski RJ, Flegal KM, Campbell SM et al: Increasing prevalence of overweight among US adults. The National Health and Nutrition Examination Surveys, 1960 to 1991. JAMA 272:205-211, 1994

137. Grundy SM, Cleeman JI, Daniels SR et al: Diagnosis and management of the metabolic syndrome: an American Heart Association/National Heart, Lung, and Blood Institute Scientific Statement. Circulation 112:2735-2752, 2005

138. Wang J, He D, Zhang Q et al: Resveratrol protects against Cisplatin-induced cardiotoxicity by alleviating oxidative damage. Cancer Biother Radiopharm 24:675-680, 2009

139. Ma H, Jones KR, Guo R et al: Cisplatin Compromises Myocardial Contractile Function and Mitochondrial Ultrastructure: Role of Endoplasmic Reticulum Stress. Clin Exp Pharmacol Physiol, 2009

140. De Sutter J, De Backer J, Van de Veire N et al: Effects of age, gender, and left ventricular mass on septal mitral annulus velocity (E') and the ratio of transmitral early peak velocity to E' (E/E'). Am J Cardiol 95:1020-1023, 2005

141. Iurisci I, Tinari N, Natoli C et al: Concentrations of galectin-3 in the sera of normal controls and cancer patients. Clin Cancer Res 6:1389-1393, 2000

142. van den Belt-Dusebout AW, Nuver J, de Wit R et al: Long-term risk of cardiovascular disease in 5-year survivors of testicular cancer. J Clin Oncol 24:467-475, 2006

143. O'Leary DH, Bots ML: Imaging of atherosclerosis: carotid intima-media thickness. Eur Heart J 31:1682-1689, 2010

144. Chambless LE, Folsom AR, Davis V et al: Risk factors for progression of common carotid atherosclerosis: the Atherosclerosis Risk in Communities Study, 1987-1998. Am J Epidemiol 155:38-47, 2002

145. Howard G, Sharrett AR, Heiss G et al: Carotid artery intimal-medial thickness distribution in general populations as evaluated by B-mode ultrasound. ARIC Investigators. Stroke 24:1297-1304, 1993

146. de Groot E, Hovingh GK, Wiegman A et al: Measurement of arterial wall thickness as a surrogate marker for atherosclerosis. Circulation 109:III33-III38, 2004

147. de Leeuw K, Smit AJ, de Groot E et al: Longitudinal study on premature atherosclerosis in patients with systemic lupus erythematosus. Atherosclerosis 206:546-550, 2009

148. Block JA, Sequeira W: Raynaud's phenomenon. Lancet 357:2042-2048, 2001

149. Vogelzang NJ, Torkelson JL, Kennedy BJ: Hypomagnesemia, renal dysfunction, and Raynaud's phenomenon in patients treated with cisplatin, vinblastine, and bleomycin. Cancer 56:2765-2770, 1985

150. Brydoy M, Oldenburg J, Klepp O et al: Observational study of prevalence of long-term Raynaud-like phenomena and neurological side effects in testicular cancer survivors. J Natl Cancer Inst 101:1682-1695, 2009
151. Glendenning JL, Barbachano Y, Norman AR et al: Long-term neurologic and peripheral vascular toxicity after chemotherapy treatment of testicular cancer. Cancer 116:2322-2331, 2010
152. Cheng KS, Tiwari A, Boutin A et al: Differentiation of primary and secondary Raynaud's disease by carotid arterial stiffness. Eur J Vasc Endovasc Surg 25:336-341, 2003
153. Wethal T, Haugnes HS, Kjekshus J et al: C-reactive protein; a potential marker of second cancer and cardiovascular disease in testicular cancer survivors? Eur J Cancer 46:3425-3433, 2010
154. Sagstuen H, Aass N, Fossa SD et al: Blood pressure and body mass index in long-term survivors of testicular cancer. J Clin Oncol 23:4980-4990, 2005
155. Eckel RH, Grundy SM, Zimmet PZ: The metabolic syndrome. Lancet 365:1415-1428, 2005
156. Despres JP, Lemieux I: Abdominal obesity and metabolic syndrome. Nature 444:881-887, 2006
157. Haugnes HS, Aass N, Fossa SD et al: Predicted cardiovascular mortality and reported cardiovascular morbidity in testicular cancer survivors. J Cancer Surviv 2:128-137, 2008
158. Oterdoom LH, de Vries AP, Gansevoort RT et al: Fasting insulin modifies the relation between age and renal function. Nephrol Dial Transplant 22:1587-1592, 2007
159. Lambers Heerspink HJ, Brantsma AH, de Zeeuw D et al: Albuminuria assessed from first-morning-void urine samples versus 24-hour urine collections as a predictor of cardiovascular morbidity and mortality. Am J Epidemiol 168:897-905, 2008
160. 1993 guidelines for the management of mild hypertension. Memorandum from a World Health Organization/International Society of Hypertension meeting. Guidelines Subcommittee of the WHO/ISH Mild Hypertension Liaison Committee: Hypertension 22:392-403, 1993
161. Gietema JA, Sleijfer DT, Willemse PH et al: Long-term follow-up of cardiovascular risk factors in patients given chemotherapy for disseminated nonseminomatous testicular cancer. Ann Intern Med 116:709-715, 1992
162. Calle EE, Kaaks R: Overweight, obesity and cancer: epidemiological evidence and proposed mechanisms. Nat Rev Cancer 4:579-591, 2004
163. Laaksonen DE, Niskanen L, Punnonen K et al: Testosterone and sex hormone-binding globulin predict the metabolic syndrome and diabetes in middle-aged men. Diabetes Care 27:1036-1041, 2004
164. Kupelian V, Page ST, Araujo AB et al: Low sex hormone-binding globulin, total testosterone, and symptomatic androgen deficiency are associated with development of the metabolic syndrome in nonobese men. J Clin Endocrinol Metab 91:843-850, 2006
165. Haring R, Volzke H, Felix SB et al: Prediction of metabolic syndrome by low serum testosterone levels in men: results from the study of health in Pomerania. Diabetes 58:2027-2031, 2009
166. Yeap BB, Chubb SA, Hyde Z et al: Lower serum testosterone is independently associated with insulin resistance in non-diabetic older men: the Health In Men Study. Eur J Endocrinol 161:591-598, 2009
167. Van Gaal LF, Mertens IL, De Block CE: Mechanisms linking obesity with cardiovascular disease. Nature 444:875-880, 2006
168. Croce K, Libby P: Intertwining of thrombosis and inflammation in atherosclerosis. Curr Opin Hematol 14:55-61, 2007
169. Lorenz MW, Markus HS, Bots ML et al: Prediction of clinical cardiovascular events with carotid intima-media thickness: a systematic review and meta-analysis. Circulation 115:459-467, 2007
170. Katsube Y, Saro H, Naka M et al: Decreased baroreflex sensitivity in patients with stable coronary artery disease is correlated with the severity of coronary narrowing. Am J Cardiol 78:1007-1010, 1996
171. Gianaros PJ, Jennings JR, Olafsson GB et al: Greater intima-media thickness in the carotid bulb is associated with reduced baroreflex sensitivity. Am J Hypertens 15:486-491, 2002
172. Dirix LY, Libura M, Libura J et al: In vitro toxicity studies with mitomycins and bleomycin on endothelial cells. Anticancer Drugs 8:859-868, 1997
173. Ito H, Okafuji T, Suzuki T: Vitamin E prevents endothelial injury associated with cisplatin injection into the superior mesenteric artery of rats. Heart Vessels 10:178-184, 1995
174. Brouwers EE, Huitema AD, Beijnen JH et al: Long-term platinum retention after treatment with cisplatin and oxaliplatin. BMC Clin Pharmacol 8:7, 2008
175. Montiel M, Urso L, de la Blanca EP et al: Cisplatin reduces endothelial cell migration via regulation of type 2-matrix metalloproteinase activity. Cell Physiol Biochem 23:441-448, 2009

176. Ades EW, Candal FJ, Swerlick RA et al: HMEC-1: establishment of an immortalized human microvascular endothelial cell line. J Invest Dermatol 99:683-690, 1992

177. Subramanian A, Tamayo P, Mootha VK et al: Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A 102:15545-15550, 2005

178. Ago T, Sadoshima J: GDF15, a cardioprotective TGF-beta superfamily protein. Circ Res 98:294-297, 2006

179. Eggers KM, Kempf T, Lagerqvist B et al: Growth-differentiation factor-15 for long-term risk prediction in patients stabilized after an episode of non-ST-segment-elevation acute coronary syndrome. Circ Cardiovasc Genet 3:88-96, 2010

180. Wollert KC, Kempf T, Peter T et al: Prognostic value of growth-differentiation factor-15 in patients with non-ST-elevation acute coronary syndrome. Circulation 115:962-971, 2007

181. Brown DA, Breit SN, Buring J et al: Concentration in plasma of macrophage inhibitory cytokine-1 and risk of cardiovascular events in women: a nested case-control study. Lancet 359:2159-2163, 2002

182. Kempf T, Eden M, Strelau J et al: The transforming growth factor-beta superfamily member growth-differentiation factor-15 protects the heart from ischemia/reperfusion injury. Circ Res 98:351-360, 2006

183. Huang CY, Beer TM, Higano CS et al: Molecular alterations in prostate carcinomas that associate with in vivo exposure to chemotherapy: identification of a cytoprotective mechanism involving growth differentiation factor 15. Clin Cancer Res 13:5825-5833, 2007

184. Fu M, Zhu X, Zhang J et al: Egr-1 target genes in human endothelial cells identified by microarray analysis. Gene 315:33-41, 2003

185. Hamdi M, Popeijus HE, Carlotti F et al: ATF3 and Fra1 have opposite functions in JNK- and ERK-dependent DNA damage responses. DNA Repair (Amst) 7:487-496, 2008

186. Nawa T, Nawa MT, Adachi MT et al: Expression of transcriptional repressor ATF3/LRF1 in human atherosclerosis: colocalization and possible involvement in cell death of vascular endothelial cells. Atherosclerosis 161:281-291, 2002

187. Kool J, Hamdi M, Cornelissen-Steijger P et al: Induction of ATF3 by ionizing radiation is mediated via a signaling pathway that includes ATM, Nibrin1, stress-induced MAPkinases and ATF-2. Oncogene 22:4235-4242, 2003

188. Chen SC, Liu YC, Shyu KG et al: Acute hypoxia to endothelial cells induces activating transcription factor 3 (ATF3) expression that is mediated via nitric oxide. Atherosclerosis 201:281-288, 2008

189. Cai Y, Zhang C, Nawa T et al: Homocysteine-responsive ATF3 gene expression in human vascular endothelial cells: activation of c-Jun NH(2)-terminal kinase and promoter response element. Blood 96:2140-2148, 2000

190. Kawauchi J, Zhang C, Nobori K et al: Transcriptional repressor activating transcription factor 3 protects human umbilical vein endothelial cells from tumor necrosis factor-alpha-induced apoptosis through down-regulation of p53 transcription. J Biol Chem 277:39025-39034, 2002

191. Nobori K, Ito H, Tamamori-Adachi M et al: ATF3 inhibits doxorubicin-induced apoptosis in cardiac myocytes: a novel cardioprotective role of ATF3. J Mol Cell Cardiol 34:1387-1397, 2002

192. Eckstein N, Servan K, Girard L et al: Epidermal growth factor receptor pathway analysis identifies amphiregulin as a key factor for cisplatin resistance of human breast cancer cells. J Biol Chem 283:739-750, 2008

193. van den Belt-Dusebout AW, Nuver J, de Wit R et al: Long-term risk of cardiovascular disease in 5-year survivors of testicular cancer. J Clin Oncol 24:467-475, 2006

194. de Haas EC, Zwart N, Meijer C et al: Association of PAI-1 gene polymorphism with survival and chemotherapy-related vascular toxicity in testicular cancer. Cancer 15:5628-36, 2010

195. Oldenburg J, Kraggerud SM, Cvancarova M et al: Cisplatin-induced long-term hearing impairment is associated with specific glutathione s-transferase genotypes in testicular cancer survivors. J Clin Oncol 25:708-714, 2007

196. Oldenburg J, Kraggerud SM, Brydoy M et al: Association between long-term neuro-toxicities in testicular cancer survivors and polymorphisms in glutathione-s-transferase-P1 and -M1, a retrospective cross sectional study. J Transl Med 5:70, 2007

197. Zhou Q, Liao JK: Pleiotropic effects of statins. - Basic research and clinical perspectives -. Circ J 74:818-826, 2010

198. Papanas N, Maltezos E: Oral antidiabetic agents: anti-atherosclerotic properties beyond glucose lowering? Curr Pharm Des 15:3179-3192, 2009

199. de Wit R: Refining the optimal chemotherapy regimen in good prognosis germ cell cancer: interpretation of the current body of knowledge. J Clin Oncol 25:4346-4349, 2007

200. Bosl GJ, Geller NL, Bajorin D et al: A randomized trial of etoposide + cisplatin versus vinblastine + bleomycin + cisplatin + cyclophosphamide + dactinomycin in patients with good-prognosis germ cell tumors. J Clin Oncol 6:1231-1238, 1988

201. Blaauwbroek R, Tuinier W, Meyboom-de Jong B et al: Shared care by paediatric oncologists and family doctors for long-term follow-up of adult childhood cancer survivors: a pilot study. Lancet Oncol 9:232-238, 2008

202. Oeffinger KC, Mertens AC, Sklar CA et al: Chronic health conditions in adult survivors of childhood cancer. N Engl J Med 355:1572-1582, 2006